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13. ABSTRACT *(Maximum 200 Words)*

We have evidence that the nuclear matrix protein HET might represent an important tumor suppressor gene in human breast cancer: 1. Overexpression of HET inhibited growth. 2. It was negatively associated with S-phase fraction in breast tumors, and 16% of breast tumors did not express HET. 3. Western Blot analysis in breast tumors led to the detection of smaller products, presumably representing truncated HET proteins. 4. HET maps to a locus on chromosome 19P13 where we detected an unusually high rate of loss of heterozygozity. In the first specific aim we will directly answer whether HET is the tumor suppressor gene by performing additional LOH analysis and mutational analysis of HET in breast cancer cell lines as well as in tumors. In the second specific aim we will perform functional analysis of discovered HET mutations in breast cancer cell lines. If HET is the tumor suppressor gene at 19p13, this will have a direct impact on the mechanistic understanding of tumor suppressor genes in breast cancer, as this region has an extremely high LOH rate in human breast cancer. If our hypothesis is true, then mutational analysis of HET could become a very informative tool for breast cancer prognosis and therapy..

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INTRODUCTION

As presented in our original grant proposal, we have evidence that the nuclear matrix protein HET might represent an important tumor suppressor gene in human breast cancer: 1. Overexpression of HET inhibited growth. 2. It was negatively associated with S-phase fraction in breast tumors, and 16% of breast tumors did not express HET. 3. Western Blot analysis in breast tumors led to the detection of smaller products, presumably representing truncated HET proteins. 4. HET maps to a locus on chromosome 19p13 where we detected an unusually high rate of loss of Heterozygozity (LOH). Excited by this preliminary observation, we proposed to examine whether HET is the tumor suppressor gene at 19p13. The proposed analyses include further LOH analysis, mutational studies, as well as functional studies of the protein in breast cancer cells.

BODY

EXPERIMENTAL METHODS AND PROCEDURES

Polymerase chain reaction (PCR), Single strand chain polymorphism (SSCP), and Sequencing

Our PCR reactions are carried out on a DNA Thermal Cycler 480 (Perkin Elmer). PCR reactions for SSCP analysis are carried out using RT-PCR products from different breast cancer cell lines. We followed the protocol by Orita et al (Orita *et al.*, 1989) with minor changes: PCR reactions are performed in 30 µl volume including 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 200 µM of each dNTP, 0.13 µM of each primer, 1.3-1.5 mM $MgCl_2$ and 0.5 µl Taq polymerase (PE). 5 µl of 1:24 diluted ^{32}P-dCTP will be used for incorporation of radiolabeled dNTP. The reactions are denatured at 94°C for 2 min, followed by 30-34 repetitive steps of 1 min at 94°C, 45 " at 55-60°C, 1 min at 72°C, and the last extension step at 72°C for 5 min. 1µl of the PCR reaction will be denatured in 9 µl denaturing solution (95% deionized formamide, 10 mM NaOH, 0.25% xylene cyanol, 0.25% bromphenol blue) at 95°C for 4 min, chilled on ice for 2 min, and 4 µl are loaded onto a non-denaturing MDE (AT Biochem) gel. Gels are run at two different conditions to maximize our sensitivity for detection: at 4°C without glycerol at 40 W for 4-5 h or at room temperature with 5% glycerol at 15 W overnight. The gels will be transferred to whatman paper, dried, and exposed to X-ray film. Bands will altered mobility will be cut out from the gel, cloned and sequenced.

For the PCR of the HET exons from LCM microdissected material we use genomic DNA in a 15 µl reaction using 0.1 nmol of each primer, 100 ?M of each dNTP, 3 mM MgCl2, 1 mM spermidine, and 0.5 U Platinum Taq polymerase (GIBCO). The single band PCR products are purified using QantumPrep PCR Kleen Spin Columns (BioRad).

At the beginning of the project we performed the sequencing reactions in our lab utilizing a kit from USB, following the manufacturer's protocol. Due to the increasing number of samples to be sequenced we decided to use the sequencing core at Baylor College of Medicine/ Department of Cell Biology/Dr. Lawrence Chan for future sequencing studies.

Fluorescence in situ hybridization (FISH)

A Bac clone spanning the D19S216 locus was ordered from Research Genetics (RP11-801D6), and used as a probe. The FISH analysis was performed by Dr. Pulivarthi Rao (Baylor College of Medicine) (Iida *et al.*, 1999).

RESULTS –YEAR 2

Task1:	1-10	Sequencing Analysis
Task 2:	8-12	Fluorescence in situ hybridization

1. HET/SAF-B is mutated in breast cancer cell lines as well as in human breast tumors

First we analyzed transcripts from MCF-7/MG, T47D, and MDA-MB-468 breast cancer cell lines. RT-PCR amplification followed by subcloning of the PCR product and sequencing led to the identification of three point mutations changing amino acids (see Table II in the attached manuscript). The presence of these mutations was confirmed by direct sequencing of genomic DNA from the cell lines. To further the search for mutations, we have started PCR-amplifying genomic DNA from the nondeleted allele in the LOH-positive tumors, and the results are also shown in Table II. Two point mutations were identified which resulted in amino acid changes, and which were, most importantly, not found in the adjacent normal tissue. Our sequence analysis so far encumbered only approximately 10%, 14%, and 13% of the HET/SAFB exon

sequence from 11, 15, and 2 tumors, respectively. Although further sequence analysis might lead to the identification of additional mutations in those tumors, our preliminary results indicate that the mutation rate is not very high. It is possible that other epigenetic changes might play a role in inactivating SAFB. Inactivations of tumor suppressor genes through methylation (Esteller *et al.*, 2000; Merlo *et al.*, 1995; Simpson *et al.*, 2000), through altered ubiquitin degradation (Pagano *et al.*, 1995; Scheffner, 1998; Tam *et al.*, 1997; Zaika *et al.*, 1999), and through mislocalization (Chen *et al.*, 1995) are increasingly been recognized as alternative inactivating mechanisms. Our own western blot analyses have demonstrated variations in the abundance of SAFB in breast tumor specimens, and, in 16% of the tumors (10 of 61), no protein was detectable even after prolonged exposure of X-ray films (Townson et al. Clinical Cancer Research in press, preprint is attached). Thus, other inactivating mechanisms might indeed be involved in loss of HET/SAFB. We have started a collaboration with Dr. Sarah Sukumar (Johns Hopkins University) who has recently identified that Hox5a is hypermethylated in breast cancer which in turn results in decreased p53 expression (Raman *et al.*, 2000). We have already designed primer pairs, and we will analyze the methylation status of the HET/SAF-B promoter in the near future.
It is however also possible that HET/SAF-B is not the major mutational target at 19p13, or that the locus harbors more than one candidate gene. This sort of clustering has been seen in other regions of high LOH; e.g. a very small region on chromosome 13q (~25 cM) harbors a series of interesting genes including BRCA2, Brush1, FKHR, and Rb. Therefore we will also study the high LOH region on chromsome 19p13 in more detail, and analyze whetehr other genes could be involved as potential tumor suppressor genes. In a recently awarded DOD Concept Grant we proposed to use a new method to rapidly screen the 19p13 region for other candidate suppressor genes. We proposed to identify additional tumor suppressor candidate genes using an adaptation of cDNA microarray technology to evaluate DNA copy number (Pollack *et al.*, 1999). ESTs from the region of high LOH (19p13.2-3) will be arrayed onto filters, and candidates will be identified based on decreases in DNA copy number (loss of one allele, or homozygous loss) in breast tumors.

2. The 19p13 locus is a frequent target for translocations in breast cancer cell lines

In order to identify a cell line which can serve as a model for HET haploinsufficiency, we performed FISH analysis using a number of breast cancer cell lines. LOH analysis can't be performed since it is impossible to differentiate between actual loss and noninformative due to the absence of a control. For the FISH analysis we initiated a collaboration with Dr. P. Rao here at Baylor College of Medicine, who is an expert in cytogenetics. We found that out of 4 cell lines only one has a "normal" 19p13 locus (T47D). The other cell lines have either lost one copy (MDA-435), or show amplification combined with translocation (MCF-7 and MDA-MB-231).

Thus, we have successfully performed and partially finished Tasks 1 and 2 for year 2. We have also started two very promising collaborations with outstanding investigators (Drs Sukumar and Rao).

DISCUSSION

After having confirmed the extremely high LOH rate in year 1 of the award, we have now begun to sequence the remaining HET/SAFB allele in breast tumors. We have already identified mutations, which indeed make HET/SAFB a potential tumor suppressor gene in human breast cancer. However, according to our preliminary results the mutation rate might not be very high. Therefore we have decided to study methylation of the gene, a mechanism which has recently been shown to be involved in tumor suppressor gene inactivation. In addition we will study the

region on chromosome 19p13 in more detail using an adaptation of cDNA array technology, and we have recently received funding to perform these studies.

The relocation of this project to Baylor College of Medicine Breast Center in year 1 of the award has had a positive impact upon the project. We have made significant progress, and we were also able to publish two manuscripts during the last year (Townson *et al.*, 2000) (Oesterreich *et al.*, 2000). One manuscript has been resubmitted with minor changes (see Appendix).

KEY RESEARCH ACCOMPLISHMENTS

- We have confirmed nucleotide changes in HET in a breast cancer cell line.
- We have detected mutations in HET in breast tumors but not in the adjacent normal tissue.
- We have found a number of CG islands in the HET/SAFB promoter, and we have designed primers to study methylation as a mechanism of inactivation.

REPORTABLE OUTCOMES

Publications:

1. **Oesterreich S**, Zhang Q, Hopp T, Fuqua SAW, Michaelis M, Zhao HH, Davie JR, Osborne CK, Lee AV. Estrogen Receptor bound to the antiestrogen Tamoxifen strongly interacts with the nuclear matrix protein HET/SAF-B, a novel inhibitor of estrogen receptor-mediated transactivation. Mol. Endo. 14, 369-381, 2000.
2. **Oesterreich S**, D. Craig Allred, Syed Mohsin, Adrian V. Lee, C. Kent Osborne, Peter O'Connell· Loss of Heterozygozity at the HET/SAF-B Locus on Chromosome 19p13 in Human Breast Cancer. Resubmitted to Br. J. Cancer (see appendix)
3. Steven M. Townson, Toby Sullivan, qingPing Zhang, Gary M. Clark, C. Kent Osborne, Adrian V. Lee, and **Steffi Oesterreich.** HET/SAF-B Overexpression Causes Growth Arrest and Multinuclearity and Is Associated with Aneuploidy in Human Breast Cancer. Clinical Can Res 6, 3788-3796, 2000.

Oral Presentation:

DOD Breast Cancer Research Program Meeting Era of Hope, Atlanta, 6/2000
Oesterreich, Zhang, Lee, O'Connell: The nuclear matrix protein HET/SAFB is a potential tumor suppressor gene in human breast caner.

Funding:

DOD Concept Award: Rapid Screen for Tumor Suppressor Genes on Chromosome 19p13
$50,000, 07/01/2000-06/30/2001

Postdoctoral Fellowship for Dr. M. Ivanova (DOD): HET/SAF-B as a tumor suppressor gene in human breast cancer. Generation of a mouse model.

Employment opportunity:

I accepted a new position as an Assistant Professor at Baylor College of Medicine (BCM) during year 1 of the award. This relocation has had a very positive impact of my research productivity.

CONCLUSIONS

We have made further progress, and there are no major modifications to the experimental plans. We are a step further in proofing that HET/SAF-B could indeed be a tumor suppressor gene in human breast cancer. The progress has also allowed us to submit other grants to support further analysis of HET/SAF-B (e.g. DOD Postdoctoral Fellowship for the generation of a HET/SAF-B knockout mouse). We are however aware that there might be other candidate genes in this area of extremely high LOH, and we will start to decipher this chromosomal locus in more detail using adapted array technology and DNA from breast tumors with and without LOH. We have recently received funding to perform these studies.

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APPENDICES

1. **Oesterreich S** et al.: Estrogen Receptor bound to the antiestrogen Tamoxifen strongly interacts with the nuclear matrix protein HET/SAF-B, a novel inhibitor of estrogen receptor-mediated transactivation. Mol. Endo. 14, 369-381, 2000.
2. **Oesterreich S** et al.: Loss of Heterozygozity at the HET/SAF-B Locus on Chromosome 19p13 in Human Breast Cancer. Resubmitted to Br. J. Cancer (see appendix)
3. Townson et al.: HET/SAF-B Overexpression Causes Growth Arrest and Multinuclearity and Is Associated with Aneuploidy in Human Breast Cancer. Clinical Can Res 6, 3788-3796, 2000.

HIGH LOSS OF HETEROZYGOSITY AT THE HET/SAF-B LOCUS ON CHROMOSOME 19P13 IN HUMAN BREAST CANCER

Running Title: LOH on Chromosome 19p13

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Abstract

We have recently discovered that the nuclear matrix protein HET/SAF-B is an estrogen receptor corepressor. Since it has become clear that many steroid receptor cofactors play important roles in breast tumorigenesis, we asked whether HET/SAF-B could also be involved in breast cancer. To address this question, we determined whether losses or alterations in the gene locus and in the gene itself are frequent in breast cancer tissue. We used laser capture microdissection for isolating DNA from paired primary breast tumor and normal tissue specimens, and we determined the loss of heterozygosity (LOH) at chromosome 19p13.2-3 by use of microsatellite markers. We detected LOH at the marker D19S216, which colocalizes with HET/SAF-B locus, in specimens from 29 (78.4%) of 37 informative patients. By use of additional markers we concluded that the peak LOH rate occurred at the HET/SAF-B locus, with LOH frequencies ranging from 21.6% to 47.2% at other markers. The finding of a very high LOH rate at the marker D19S216 strongly indicates the presence of a breast tumor-suppressor gene locus. Preliminary findings of mutations in HET/SAF-B suggest that this indeed may be a promising candidate.

Keywords: loss of heterozygosity, chromosome19p13, breast cancer, HET/SAF-B, tumor suppressor gene, mutation

Abbreviations: LOH, loss of heterozygosity; HET/SAF-B, hsp27-ERE-TATA-binding protein/scaffold attachment factor B

Introduction

The estrogen receptor (ER) is a nuclear steroid receptor that upon activation by its ligands (e.g. estrogen) initiates a cascade of events resulting in increased cellular proliferation in its target tissues (Warner *et al.*, 1999). Since estrogen is one of the most potent mitogens for breast cancer cells, it is no surprise that ER is the most important target for endocrine therapy of breast cancer (Osborne, 1998). Recently, a number of factors, which regulate nuclear hormone receptor activity, have been identified. Cofactors capable of increasing receptor action, termed coactivators, include transcriptional intermediary factor 1 (TIF1), nuclear receptor interacting protein 1 (NRIP1), nuclear receptor coactivator 2 (TIF2), steroid receptor coactivator 1 (SRC1), amplified in breast cancer 1 (AIB1), the cyclic AMP (cAMP)-response element binding protein (CREB) binding protein (CBP) (Glass *et al.*, 1997; Shibata *et al.*, 1997), and many more. The family of corepressors (negative regulators) of ER is smaller; the best characterized ones being the nuclear receptor corepressor (N-CoR) (Horlein *et al.*, 1995; Shibata *et al.*, 1997) the silencing mediator of retinoid and thyroid receptors (SMRT) (Chen & Evans, 1995; Sande & Privalsky, 1996) and the repressor of ER activity (REA) (Montano *et al.*, 1999). The overexpression of coactivators or the loss of corepressors could lead to unregulated estrogen-dependent pathways related to mammary epithelial cell proliferation, and thus to breast tumorigenesis. And indeed, some of the ER cofactors have recently been characterized as playing major roles in breast tumorigenesis (Anzick *et al.*, 1997; Horlein *et al.*, 1995; Shibata *et al.*, 1997). The ER coactivator AIB1 was cloned during a search on the long arm of chromosome 20 for genes whose expression and copy number are elevated in human breast cancer, and subsequent analysis in 105 breast tumor specimens confirmed its overexpression (Anzick *et al.*, 1997). Interestingly, the tumor suppressor gene BRCA1 has recently been characterized as an ER corepressor (Fan *et*

al., 1999) again suggesting that ER coregulators are crucial in breast tumorigenesis. Thus, it is expected that other ER coactivators and corepressors might play similar important roles in breast cancer development and progression.

The nuclear matrix protein HET/SAF-B (Oesterreich *et al.*, 1997; Renz & Fackelmayer, 1996) has been shown to be an ER corepressor (Oesterreich *et al.*, 2000). ER and HET/SAF-B interact in in vitro binding assays (Glutathione-S-Transferase [GST]-pulldown assays) and in cell lines (co-immunoprecipitation experiments). In cell lines, there is binding of HET/SAF-B to ER in the presence or absence of estradiol; however, binding is significantly increased by the antiestrogen tamoxifen. Overexpression of HET/SAF-B results in repression of estrogen-mediated transactivation of gene expression by the ER. Furthermore, as a result of HET/SAF-B overexpression, the antagonist activity of tamoxifen on ER can be enhanced, and the agonist activity of tamoxifen can be inhibited.

These results led us to investigate whether the ER corepressor HET/SAF-B could also be involved in breast tumorigenesis. Towards this goal we analyzed whether the chromosomal locus for HET/SAF-B is a frequent target for chromosomal aberrations, i.e., allelic deletion. Allelic deletion manifested as loss of heterozygosity (LOH) at polymorphic loci is recognized as a hallmark for genes involved in tumor suppression; thus, high LOH at the HET/SAF-B locus would suggest that this recently identified ER cofactor could play an important role in breast tumor suppression. In the present study we proposed to study human breast cancer specimens for the rate of LOH at different markers that colocalize with or are adjacent to the HET/SAF-B locus on chromosome 19p13. To strengthen our hypothesis we also performed mutational analysis of HET/SAF-B in both LOH-positive tumors as well as in breast cancer cell lines.

Methods

Patients, Tissues, and Microdissection: The 57 patients whose tissue was evaluated in this study had primary breast cancer; their archival paraffin-embedded tissues were used for the analysis. For 52 of the 57 patients, a single paraffin section yielded sufficient normal tissue (terminal duct lobular unit) and primary cancer. For five patients, normal lymph node tissues were recovered from separate blocks. Single 5 μm sections were cut from the selected blocks, mounted on glass slides, deparaffinized, and lightly counterstained with nuclear fast red to guide laser capture microdissection (LCM) of cells using an LCM instrument (Pixcell by Arcturus Engineering) (Emmert-Buck *et al.*, 1996; Simone *et al.*, 1998). Briefly, a transparent thermoplastic film (ethylene vinyl acetate polymer) was placed over the section on the slides. A laser directed through the microscope optics was activated, causing the thermoplastic film to melt and fuse with the underlying targeted cells. The selected cells remained adherent to the film when it was removed from the slide. An average of approximately 1,000 cells (about 100 cell clusters of 10 cells each) was harvested from each tissue sample.

LOH analysis: LOH analysis was performed as recently described (O'Connell *et al.*, 1999). Briefly, DNA was prepared by a modification of the method of Wright and Manos (Wright & Manos, 1990). The embedded cells were incubated for 18-20 hours at 37° C in 60 μL of a lysis buffer that contained of 10mM Tris-HCl (pH 8.5), 1mM EDTA, 0.045% NP-40, 0.045% Tween-20, and 1.0 mg/mL proteinase K. The proteinase was then inactivated at 95°C for 10 minutes. PCR and gel electrophoresis was performed as described previously by us (O'Connell *et al.*, 1999). Samples were evaluated for LOH using the microsatellite markers D19S216, D19S413, D19S591, and D19S883. The primer pairs were obtained from Research Genetics, Inc. (Birmingham, AL). Mapping data were obtained from the Genome DataBase (GDBTM) at Johns

Hopkins University (Fasman *et al.*, 1997; Talbot & Cutichia, 1999). The intensity ratios of bands in electrophoretic gels representing different marker alleles in the DNA obtained from paired normal and breast cancer tissues were calculated from digitized data collected with a storage phosphor device and analyzed with the Molecular Dynamics ImageQuant software package (Molecular Dynamics, Sunnyvale, CA). LOH was considered positive when the proportion [(tumor allele 1/tumor allele 2)/(normal allele 1/normal allele 2)] equaled either less than 0.71 (tumor allele 1 LOH) or greater than 1.4 (tumor allele 2 LOH).

Mutational Analysis: RNA from MCF-7/MG (Oesterreich *et al.*, 1993), T47D, and MDA-MB-468 breast cancer cell lines was isolated using Qiagen RNeasy kit (Valencia, CA) according to the manufacturer's instructions. First strand HET/SAF-B cDNAs were synthesized in two parts (a 5' and 3' segment) by reverse transcriptase(RT)-PCR using Avian Myeoblastosis Virus (AMV) RT (Promega, Madison, WI) on 1ug of total RNA as previously described (Wang *et al.*, 1999) (reference ok?). The primers for RT were 5'-GAGTCTCTTGACTTCCGAGGC-3' (for 5' fragment) and 5'-TCCAAGTACTCAGTAGCGGCG-3' (for 3' fragment). Multiple PCR primers were designed to amplify overlapping regions covering the total cDNA (see footnotes for Table II). The amplified PCR products were cloned using a TA cloning kit (Invitrogen, Carlsbad, CA), and DNA was isolated and sequenced from at least two clones using Quiaprep Miniprep kit (Quiagen, Valencia, CA).

To analyze the genomic DNA from the LOH-positive tumors, we have started mapping the exon/intron structure of the human HET/SAF-B gene by PCR and sequencing. So far we have identified 10 exons, and we designed primer pairs to partially amplify three exons (1F: 5'ATGGCGAGAGGACGGACT-3" and 1R (intronic): 5'-gcgtctggtctaaaactgagaa-3', product size=271bp; QP1F: 5'-GACTCTGTCAGGCCTAGGTGATTC-3' and QP1R: 5'-

GCTTCATCCAACACACTGATATCC-3', product size 401bp; QP6F: 5'-GAGCTTCCAAAAGCCAGGATCGC-3' and QP6R: 5'-CGCTCCTGCTCATAGCGCAGTT-3', product size=364bp). We analyzed 11 tumors with 1F/R, 15 tumors with QP1F/R, and 2 tumors with QP6F/R. The PCR products were cleaned using Quiaquick PCR purification kit (Quiagen), and directly sequenced. The PCR was performed twice, and the product sequenced from both orientations.

Sequencing: The sequence of cDNA was determined using an Applied Biosystems model 310 genetic analyzer.

Statistical analysis: The confidence intervals were calculated with the expression $1.96\sqrt{p\times(1-p)/i}$, where p = the LOH frequency and i = number of informative patients (Dawson Saunders & TRapp, 1994).

Results

We have previously assigned HET/SAF-B to chromosome 19-band p13.2-13.3 (DuPont *et al.*, 1997). Markers have been positioned on chromosome 19p by a combination of genetic linkage mapping (in centimorgans or cM) and/or radiation hybrid mapping (in centirays or cR). Several anchor markers are mapped by both methods and 1 cM approximates 2 cR on this map (Deloukas *et al.*, 1998). On the chromosome 19 radiation hybrid map (Deloukas *et al.*, 1998) HET/SAF-B is positioned at 34.7 cRays. The polymorphic marker D19S216 has been placed on both maps (20.1 cM, 35.9 cRays), so that HET-SAF-B maps in the D19S591-D19S216 interval just proximal to D19S216. We tested this region for LOH using D19S216 and a series of additional markers spanning the chromosome band 19p13, namely, D19S884 (5.5 cM), D19S591 (9.8 cM), D19S216 (20.1 cM), and D19S413 (31.2 cM). LOH studies were carried out by use of normal and primary breast cancer tissues from 57 patients. Three of the specimens showed evidence of microsatellite instability and were excluded from further analysis. The results of this LOH study are shown in Table 1. Marker D19S216 near HET/SAF-B showed the highest rate of LOH (78%). Figure 1 summarizes data from the subset of 25 D19S216-informative patients with interstitial LOH events. An additional 12 patients (not presented) either showed no LOH, or showed LOH for all markers. These breakpoints can map the smallest region of overlap for the LOH region(s). The majority of the patients show LOH events spanning D19S591-D19S216. Four patients (numbers 96, 179, 1086, and 1094) showed LOH events but remained heterozygous for D19S216. LOH events in four other patients (numbers 190, 207, 613, and 742) lost only D19S216, and patient 810 lost DNA sequences including D19S216 and D19S413. No D19S216-informative tumors exclusively lost D19S413. These data indicate that the interval

between D19S591-D19S216 harbors a tumor suppressor gene important in human breast cancer. As mentioned above, HET/SAF-B maps to this interval. To support our hypothesis, we performed mutational analysis of the remaining HET/SAF-B allele in the LOH-positive tumors. We also sequenced HET/SAF-B cDNA in three breast cancer cell lines.

First we analyzed transcripts from MCF-7/MG, T47D, and MDA-MB-468 breast cancer cell lines. RT-PCR amplification followed by subcloning of the PCR product and sequencing led to the identification of three point mutations changing amino acids (Table II). To further the search for mutations, we have started PCR-amplifying genomic DNA from the nondeleted allele in the LOH-positive tumors, and the results are also shown in Table II. Two point mutations were identified which resulted in amino acid changes, and which were, most importantly, not found in the adjacent normal tissue. Thus, evidence from sequence analysis of HET/SAF-B suggests that the gene indeed is a very promising candidate for a breast cancer tumor suppressor gene at the high LOH locus on chromosome 19p13.

Discussion

Several groups have performed LOH studies on chromosome 19p13. Kerangueven et al (Kerangueven *et al.*, 1997) had identified D19S216 as a marker with consistent loss (20%-30% of patients) in breast cancer using genomic DNA isolated from whole breast tumors. Bignell et al. (Bignell *et al.*, 1998) also performed an LOH study on chromosome 19p13.3, with the goal of analyzing chromosomal loss of the LKB1 gene. (The serine/threonine kinase LKB1 is mutated in patients with Peutz-Jeghers Syndrome, resulting in intestinal hamartomas associated with an elevated risk for cancer). They used the LKB-linked marker D19S565, which co-localizes with D19S883 (see Fig. 1). The Bignell study detected LOH in 7.5% of informative breast cancer

specimens, as compared with 21.6% in our study.

It is difficult to compare the LOH rates from our present LCM-based study to those of previous reports, since only a few studies using LCM material have been published. For instance Bignell et al saw 7.5% (3 of 40) LOH with D19S565 using whole tissue genomic DNA while we found 21.6% (8 of 37) using LCM material. Though part of this difference might simply reflect the small number of samples, we have previously seen that LCM enriches for tumor cells and thus always results in a higher LOH rate. As an example, we found 53% LOH (32 of 60) at D19S216 using an essentially identical set of manually microdissected archival paraffin-embedded primary breast cancer specimens (data not shown), but saw 78% LOH (29 of 37) using LCM. Brown et al. (Brown *et al.*, 1999) also noted elevated LOH rates at 8p12-22 in ovarian cancers when comparing LCM-based LOH rates with those determined in previous allelotyping studies. Tamura et al. (Tamura *et al.*, 1994) noted 35% LOH at the retinoblastoma (RB) locus on chromosome 13 from whole tumors, but a 59% rate of RB locus LOH when the tumor cells were enriched by flow sorting. We have also determined a rate of 56% LOH at the RB locus (data not shown) in our LCM-based breast cancer studies.

Our rationale for this study was that the ER corepressor HET/SAF-B might represent a new tumor suppressor gene, and our present finding would certainly support this hypothesis. LOH frequency at D19S591--HET/SAF-B—D19S216 region is among the highest yet measured in breast cancer, and mutational analysis of the HET/SAF-B gene in both human breast cancer cell lines and tumors revealed point mutations resulting in amino acid changes. Our sequence analysis so far encumbered only approximately 10%, 14%, and 13% of the HET/SAF-B exon sequence from 11, 15, and 2 tumors, respectively. Thus, further sequence analysis might lead to the identification of additional mutations in those tumors. It is also possible that other epigenetic

changes might play a role in inactivating HET/SAF-B. Inactivations of tumor suppressor genes through methylation (Esteller *et al.*, 2000; Merlo *et al.*, 1995; Simpson *et al.*, 2000), through altered ubiquitin degradation (Pagano *et al.*, 1995; Scheffner, 1998; Tam *et al.*, 1997; Zaika *et al.*, 1999), and through mislocalization (Chen *et al.*, 1995) are increasingly been recognized as alternative inactivating mechanisms. Our own western blot analyses have demonstrated variations in the abundance of HET/SAF-B in breast tumor specimens, and, in 16% of the tumors (10 of 61), no protein was detectable even after prolonged exposure of X-ray films (Towson et al, under review).

In summary, we have shown that the estrogen receptor corepressor HET/SAF-B is a candidate for a tumor suppressor at chromosome 19p13, a locus which displays unusually high LOH in breast tumors.

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Table I. Loss of Heterozygosity[1] Frequencies for Genetic Markers on Chromosome 19p13.3 in Breast Cancer Patients

Marker	Location (centimorgans)	LOH frequency = no. of patients with LOH / no. of informative patients (%); (95% confidence interval)[2]
D19S883	5.5	8/37 (21.6); 8.3 - 34.9
D19S591	9.8	17/36 (47.2); 30.9 - 63.5
D19S216	20.1	29/37 (78.4); 65.1 - 91.7
D19S413	31.2	11/35 (31.4); 16.0 - 46.8

[1] Heterozygosity is the presence of two different alleles for the genetic marker; loss of heterozygosity (LOH) is present when tumor/normal allele intensities calculated as below vary from those seen in normal tissue: LOH is present when [(tumor allele 1/tumor allele 2)/(normal allele 1/normal allele 2)] ratio is either equal to or less than 0.71 for tumor allele 1 or is equal to or greater than 1.4 for tumor allele 2.

[2] The number of LOH events observed divided by the subset of those patients out of the 54 tested whose normal DNA sample was heterozygous for the genetic marker (informative cases). LOH events cannot be detected in a patient whose normal DNA is homozygous for the genetic marker tested. The confidence interval is calculated with the expression $1.96\sqrt{p\times(1-p)/i}$, where p = the LOH frequency and i = number of informative patients (*19*).

Table II. HET/SAF-B cDNA and genomic DNA mutations in breast cancer cell lines and LOH-positive tumors.

Cell line/Case	Codon	Nucleotide change	Amino acid change
MCF-7/MG[1]	1891	AAG/AGG	Lys/Arg
T47D[2]	1391	CTC/CCC	Leu/Pro
MDA-MB-468[3]	265	AAT/GAT	Asn/Asp
Tumor #48[4]	1186	GCT/GTT	Ala/Val
Tumor #30[4]	1838	GCC/GGC	Ala/Gly

[1] The 3' first strand cDNA was amplified using forward primer 5'-GGGGTGCCTGTGATTAGTGT-3' (ecpts) and reverse primer 5'-TCAGAATGGTAGCGCTCATCC-3' (ecptz).

[2] The 3' first strand cDNA was amplified using forward primer 5'-TGGACTCTCTTCTACAACCAGAGC-3' (ecptj) and reverse primer 5'-GTCACTGTTGCTCGACTTCTCC-3' (snp1as).

[3] The 5' first strand cDNA was amplified using forward primer 5'-AATGGCGGAGACTCTGTCAGGC-3' (ecptm) and reverse primer 5'-ACAGGCTGTCTGCCTTGCTC-3' (ecph).

[4] For tumors #48 and #30, genomic DNA from microdissected tumor and adjacent normal tissue was amplified using primer pairs 1F/1R and QP6F/6R (see Methods).

Figure Legend:

Figure 1: Loss of heterozygosity (LOH) profiles in the D19S216-HET/SAF-B region. Bottom: An idiogram of chromosome 19p13.11-p13.3 detailing the region of interest and the locations of the markers tested in centimorgans (cM). Top: The LOH profiles of 25 selected patients (patient numbers: 12 to1112) informative for D19S216 with interstitial breakpoints. Data for breast cancer patient numbers 12-1112 are shown horizontally for each marker. Filled circles denote patients with LOH, open circles denote heterozygous patients (no LOH), and hatched circles show non-informative patients.

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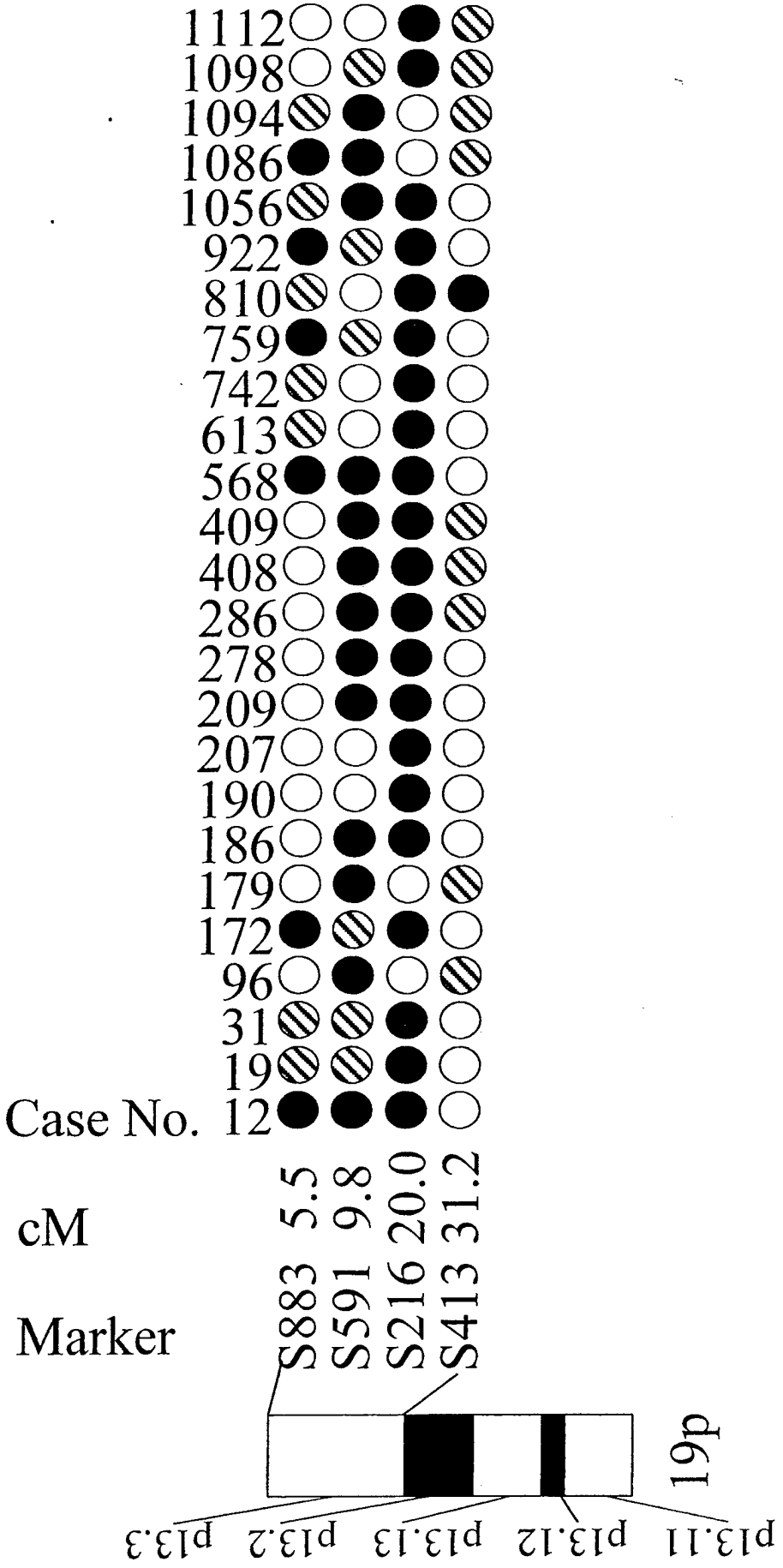
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Tamoxifen-Bound Estrogen Receptor (ER) Strongly Interacts with the Nuclear Matrix Protein HET/SAF-B, a Novel Inhibitor of ER-Mediated Transactivation

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The estrogen receptor (ER) is a ligand-dependent transcription factor that acts in a cell- and promoter-specific manner. Evidence suggests that the activity of the ER can be regulated by a number of other stimuli (*e.g.* growth factors) and that the effects of the ER are modulated by nuclear factors termed coregulators. While the interplay among these factors may in part explain the pleiotropic effects elicited by the ER, there are several other less well described mechanisms of control, such as interactions with the nuclear matrix. Here we report that the nuclear matrix protein/scaffold attachment factor HET/SAF-B is an ER-interacting protein. ER and HET/SAF-B interact in *in vitro* binding assays, with HET binding to both the ER DNA-binding domain and the hinge region. Coimmunoprecipitation experiments reveal that HET/SAF-B and ER associate in cell lines in the presence or absence of estradiol, but binding is increased by the antiestrogen tamoxifen. HET/SAF-B enhances tamoxifen antagonism of estrogen-induced ER-mediated transactivation, but at high concentrations can inhibit both estrogen and tamoxifen-induced ER activity. HET/SAF-B-mediated repression of ER activity is dependent upon interaction with the ER-DBD. While the existence of high-affinity binding sites for the ER in the nuclear matrix has been known for some time, we now provide evidence of a specific nuclear matrix protein binding to the ER. Furthermore, our data showing that HET/SAF-B binds to ER particularly strongly in the presence of tamoxifen suggests that it may be important for the antagonist effect of tamoxifen. (Molecular Endocrinology 14:369–381, 2000)

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Molecular Endocrinology

INTRODUCTION

The estrogen receptor (ER) is a member of a superfamily of nuclear transcription factors. When the ER binds estrogen it undergoes a conformational change that results in dimerization, binding to specific elements of DNA, and finally altered gene transcription (1, 2). While this model of ER action has held true for the last 30 yr, a more complete understanding has revealed that activation of the ER is extremely complex, with regulation by a diverse set of signals and nuclear factors. ER action can be altered by: 1) interaction with other nuclear transcription factors such as AP1 (3), SP1 (4–6), and members of the basal transcription machinery (1); 2) cross-talk with growth factor systems (7); and 3) associations with nuclear receptor coactivators and corepressors (8).

The existence of cofactors that can regulate the transcriptional activity of nuclear hormone receptors was first suggested by transcriptional squelching between ER and progesterone receptor (9, 10). A number of cofactors capable of increasing hormone receptor action, termed coactivators, have been identified (reviewed in Refs. 8 and 11). The family of corepressors is smaller, the best characterized being nuclear receptor corepressor (N-CoR) (8, 12) and silencing mediator of retinoid and thyroid receptors (SMRT) (13, 14). Recently, a corepressor termed REA, which is specific for

ER, has been identified (15). Many cofactors seem to regulate receptor activity by modulating chromatin structure. Coactivators such as p300/CBP (16, 17), PCAF (18, 19), and SRC-1 (20) have intrinsic histone acetyltransferase activity, which results in the destabilization of nucleosomes, creating a permissive state for promoter activation. In contrast, the corepressors N-CoR (21) and SMRT (22) associate with histone deacetylases, leading to a repressive chromatin state.

Another modulator of hormone action is the nuclear matrix, which is a dynamic structure involved in DNA replication, transcription, repair, and RNA processing (23). A role for the nuclear matrix in hormone receptor action was postulated many years ago (24–28), but only recently have specific nuclear matrix proteins been characterized that directly bind to hormone receptors and modulate their activity (29). Most recently, the glucocorticoid receptor-interacting protein GRIP 120 has been identified as the nuclear matrix protein hnRNPU (30).

HET was originally cloned in our laboratory as a nuclear matrix protein binding to the promoter of the estrogen-regulated heat shock protein hsp27 (31). Renz and Fackelmayer (32) cloned the same protein based on its ability to bind to scaffold/matrix attachment regions (S/MAR's), and hence called it scaffold attachment factor B (SAF-B). Scaffold attachment factors are a specific subset of nuclear matrix proteins that are thought to mediate the attachment of chromatin to nuclear protein structures (33, 34). A specific role for scaffold attachment factors in hormone receptor action has not been described.

HET/SAF-B has recently been shown to bind to the C-terminal domain of RNA polymerase II (RNA pol II) and to a subset of serine-/arginine-rich RNA processing factors (SR proteins) (35). This suggests that HET/SAF-B is involved in the formation of a transcriptosomal complex, bringing transcription and pre-mRNA processing together. These macromolecular complexes have previously been shown to be associated with the nuclear matrix (36, 37).

Given the recent identification of nuclear matrix factors in hormone receptor action, we asked whether the nuclear matrix protein HET/SAF-B might be involved in ER action. In this report we describe the *in vitro* and *in vivo* association of ER with HET/SAF-B, with HET/SAF-B binding the ER in both the DBD and the hinge region. The association of ER with HET/SAF-B occurs in the absence of ligand but is increased by the antiestrogen tamoxifen (Tam). HET/SAF-B can enhance the antiestrogenic effect of Tam, but when overexpressed at high levels can also repress both estrogen and Tam agonist activity on the ER. Finally, we have shown that the ER DBD is critical for the repressive activity of HET/SAF-B on ER, as HET/SAF-B does not repress activity of an ER-GAL4DBD chimera and can cause transcriptional repression of an ER DBD fused to a heterologous transcription factor. We are currently performing further studies to identify the mechanism of transcriptional repression and whether this is dependent upon the nuclear matrix properties of HET/SAF-B.

RESULTS

HET/SAF-B Binds to the ER

To analyze whether HET/SAF-B could bind to ER, we performed glutathione-*S*- transferase (GST)-pulldown experiments (Fig. 1). First we incubated *in vitro* transcribed and translated ER with full-length GST-HET/SAF-B bound to glutathione-sepharose beads (Fig. 1A). There was no signal when ER was incubated with GST only, in the absence of hormone or in the presence of estradiol (E_2). In contrast, ER interacted with GST-HET/SAF-B in the absence of hormone, in the presence of E_2, and especially in the presence of Tam. We consistently saw increased binding of ER to HET/SAF-B in the presence of Tam compared with no ligand.

Next we examined the ability of HET/SAF-B to interact with different domains of ER (represented graphically in Fig. 1B) in GST-pulldown assays. The different GST-ER domain fusion proteins were separated on SDS-PAGE and Coomassie stained, to ensure that the input of immobilized GST-fusion proteins was equal (data not shown). We examined HET/SAF-B interaction with the AF1, AF1/DNA-binding domain (DBD), DBD/Hinge, DBD, Hinge, and AF2/Hinge domains. All incubations were performed in the absence of hormone. As shown in Fig. 1C, HET/SAF-B consistently interacted strongly with the DBD/Hinge, AF2/Hinge, and AF1/DBD domains and weakly with the Hinge or DBD only. In contrast, we could not detect an interaction between HET/SAF-B and AF1. Thus, there are at least two HET/SAF-B binding sites in the ER protein, one in the DBD and the other one in the Hinge region. The HET/SAF-B interaction with AF2/Hinge was stronger than the interaction with Hinge only, suggesting that there may be another interaction domain within AF2. Thus, as described for the interaction between other cofactors and steroid receptors (38), HET/SAF-B potentially interacts with multiple regions within ER.

We next asked whether we could detect an interaction between HET/SAF-B and ER within cells. Therefore, we transiently transfected COS-7 cells with expression plasmids for HET/SAF-B and hemagglutinin (HA)-tagged ER. Immunoprecipitation of HET/SAF-B followed by immunoblotting for HA revealed a band with the molecular mass of ER (~68 kDa) that was only seen when cells were transfected with both ER and HET/SAF-B, but not in cells transfected with HET/SAF-B only (Fig. 2A, *left panel*). A similar experiment, but in a reciprocal manner, was performed using an HA-antibody to immunoprecipitate and the HET/SAF-B antibody for immunoblotting. As expected, a band at the molecular mass of HET/SAF-B (~130 kDa) was detected in cells transfected with HET/SAF-B and

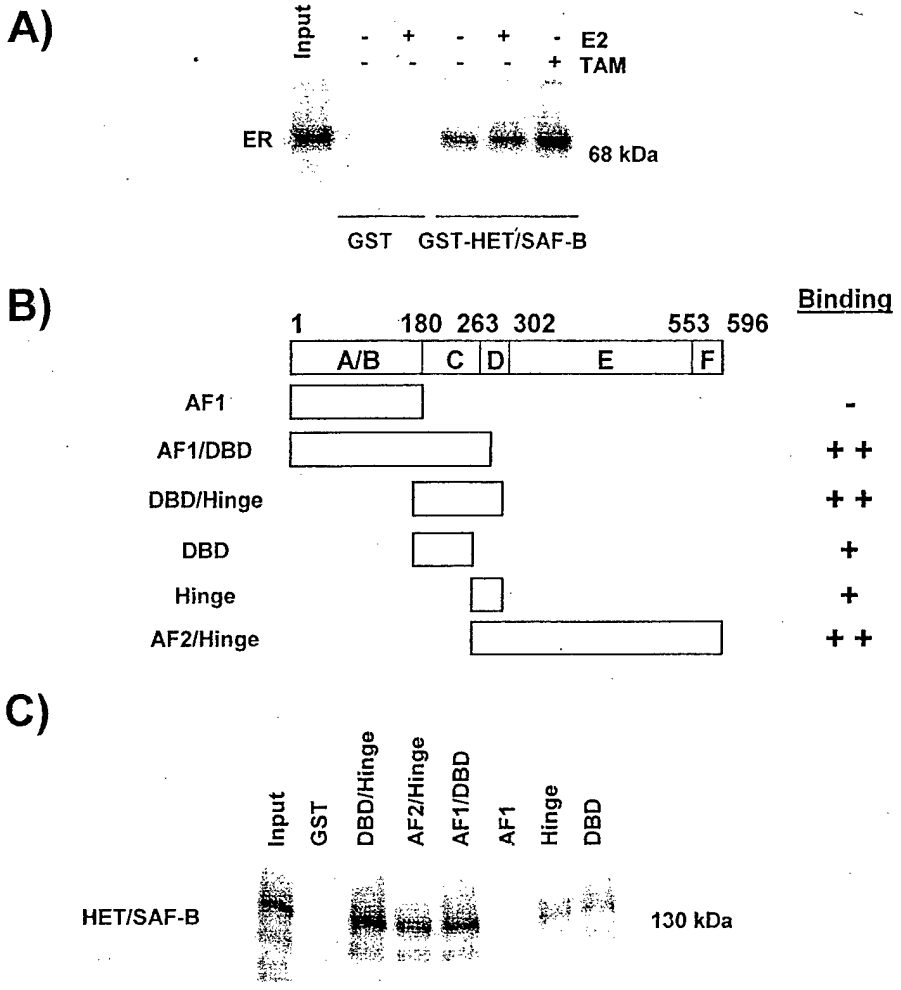

Fig. 1. HET/SAF-B Directly Interacts with ER *in Vitro* (GST-Pull-Down Experiments)

A, ER was labeled with ^{35}S-methionine by *in vitro* transcription/translation and tested for interaction with GST alone and GST-HET/SAF-B in the absence of ligands, or in the presence of 10^{-6} M E_2 or 10^{-6} M Tam (as indicated). The input lane contains 20% input of the *in vitro* transcribed/translated ER. B, Schematic presentation of GST-tagged ER domains. The numbers indicate amino acids in ER's open reading frame. C, HET/SAF-B was labeled with ^{35}S-methionine by *in vitro* transcription/translation and tested for interaction with GST alone and a number of GST-ER domain fusion proteins (as indicated). The input lane contains 20% input of the *in vitro* transcribed/translated HET/SAF-B.

ER, but not in cells transfected with HET/SAF-B only (Fig. 2A, *right panel*).

The next set of coimmunoprecipitation experiments was performed to see whether endogenous HET/SAF-B and ER indeed interact and whether this interaction was altered by E_2 or Tam. First, we immunoprecipitated HET/SAF-B from MCF-7 breast cancer cells lysed under low stringency (LS) and high stringency (HS) conditions (Fig. 2B). Under low-stringency conditions we observed coimmunoprecipitation of ER and HET/SAF-B, whereas under high-stringency conditions more HET/SAF-B was immunoprecipitated, but ER was dissociated from the complex. To demonstrate that the bands on the immunoblot are indeed antibody specific, we repeated the HET/SAF-B immunoprecipitation with HET/SAF-B antibodies preincubated with HET/SAF-B peptide, or without antibody. As shown in Fig. 2C, only the immunoprecipitation with HET/SAF-B antibody resulted in a detectable band at the molecular mass of HET/SAF-B (~130 kDa), whereas no bands were detected using a peptide-preincubated antibody or no antibody. Thus, in breast cancer cells endogenous HET/SAF-B and ER interact, and this interaction can be detected when the cells are lysed under low-stringency conditions.

To investigate the ligand dependency of this interaction, we incubated MCF-7 cells in the absence of ligand and in the presence of E_2 or Tam, and lysed them in LS buffer. After immunoprecipitation with HET/SAF-B antibodies, the membrane was immunoblotted with HET/SAF-B antibodies (Fig. 2D, *left top panel*) and ER antibodies (*left bottom panel*). While HET/SAF-B levels remained constant, coimmunoprecipitated ER levels changed. ER was detectable in the

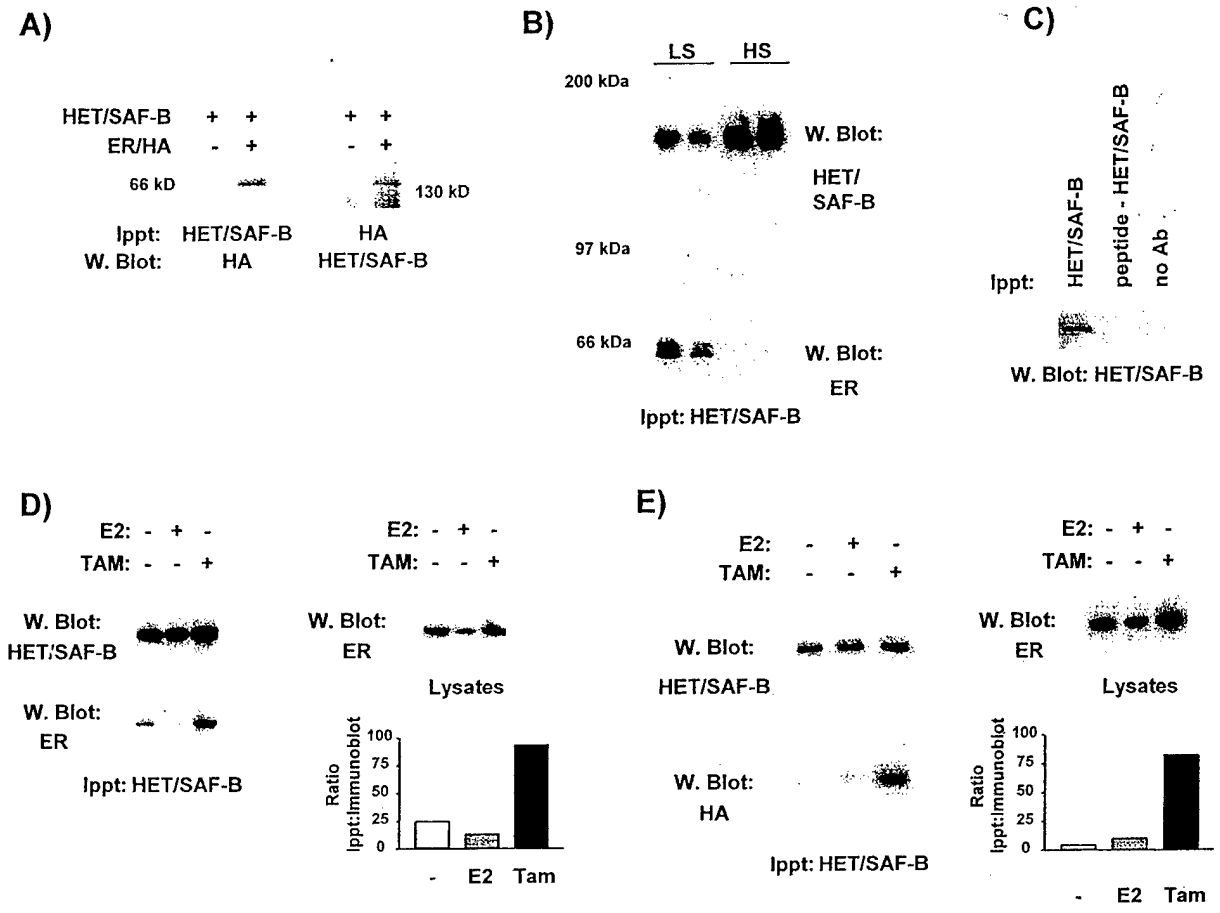

Fig. 2. HET/SAF-B and ER Interact in Cells (Coimmunoprecipitations)

A, COS-7 cells were transiently transfected with expression constructs for HA-tagged ER (ER/HA) and HET/SAF-B as indicated, and lysed in low stringency buffer. The cell lysates were immunoprecipitated with an anti-HET/SAF-B antibody, subjected to electrophoresis, and immunoblotted with an anti-HA antibody (*left panel*). The cell lysates were immunoprecipitated with an anti-HA antibody, subjected to electrophoresis, and immunoblotted with an anti-HET/SAF-B antibody (*right panel*). B, MCF-7 cells were lysed in LS and HS buffer, the lysates from duplicate plates were immunoprecipitated with an anti-HET/SAF-B antibody, and the immunoprecipitates were subjected to electrophoresis. For the immunoblot anti-HET/SAF-B and anti-ER antibody were used. C, MCF-7 LS lysates were immunoprecipitated with an HET/SAF-B antibody, with HET/SAF-B antibodies preincubated with HET/SAF-B peptide, or without antibody. The immunoblots were performed with HET/SAF-B antibody. D, MCF-7 cells were incubated without ligand, with 10^{-9} M E_2, or with 10^{-9} M Tam for 24 h. The immunoblots were performed with HET/SAF-B antibody (*left top panel*) and ER antibody (*left bottom panel*). The lysates were also immunoblotted with an ER antibody (*right top panel*). The *bar graph* represents intensity ratios of immunoprecipiated ER to immunoblotted ER in the lysates (see *Materials and Methods*). E, COS-7 cells were transfected with expression plasmids for HET/SAF-B and ER-HA. Treatment of the cells, immunoprecipitation, and immunoblotting were performed as described in panel C with the exception that HA antibody was used instead of ER antibody.

absence of ligand, low levels were detectable in the presence of E_2, but much higher levels of ER were coimmunoprecipitated in the presence of Tam. Since ER itself is known to be down-regulated by E_2 via ubiquitin-mediated degradation (39, 40), as a control we also measured ER levels in the lysate (Fig. 2D, *right top panel*). In contrast to HET/SAF-B, which did not change with E_2 and Tam treatment (data not shown), ER levels decreased dramatically after E_2 treatment but were unaffected by Tam. To account for the differences in ER levels within the actual lysates, we measured the amount of ER in the immunoprecipitate and the lysate by densitometry and presented the results as the ratio of ER levels immunoprecipiated with HET/SAF-B antibodies to ER levels in the lysate (Fig. 2D, *bar graph*). While the changes in ER levels complicate an exact quantitative analysis of the coimmunoprecipitation in the E_2-treated samples, Tam did not affect ER levels, and it can be clearly seen that ER binds more strongly to ER in the presence of Tam than in its absence (*i.e.* no ligand).

Finally, we confirmed that HET/SAF-B is strongly bound to ER in the presence of Tam by transfecting COS-7 cells with HET/SAF-B and an HA-tagged ER construct (Fig. 2E). As in MCF-7 cells, there was an association between HET/SAF-B and ER in the ab-

sence of ligand or in the presence of E_2, but again association was greater in the presence of Tam (*left bottom panel*). In cell lysates, HET/SAF-B levels did not change as a result of E_2 or Tam treatment (data not shown). However, as seen with endogenous ER in MCF-7 cells, ER levels were reduced in COS-7 cells after E_2 treatment (*right top panel*). When we corrected the changes in immunoprecipitated ER for the changes in endogenous ER levels, we were again able to detect a significant increase in the binding of HET/SAF-B to ER in the presence of Tam (Fig. 2E, *bar graph*). Thus, we conclude from our coimmunoprecipitation experiments that HET/SAF-B and ER interact, and that this interaction is stronger in the presence of the antiestrogen Tam.

HET/SAF-B Overexpression Decreases ER Activity

As shown in Fig. 2, the association between HET/SAF-B and ER is stronger in the presence of Tam as compared with no ligand. This observation prompted us to study the effect of HET/SAF-B on the antagonist activity of Tam. To do this we performed transient transfection assays in ER-negative HepG2 cells using a single estrogen response element (ERE)-tk-luciferase construct as the reporter gene (Fig. 3A). The results in Fig. 3A represent the effect of HET/SAF-B on Tam acting as an antagonist of E_2-occupied ER; *i.e.* cells were incubated in the presence of both E_2 and Tam. As expected, increasing concentrations of Tam resulted in a dose-dependent inhibition of E2-mediated ER activity (pcDNAI curve). Cotransfection of 10 ng HET/SAF-B vector did not affect E_2-mediated activation of the ER in the absence of Tam, or when Tam was added at a low concentration that does not have an antagonistic effect (10^{-10} M). In contrast, at higher concentrations of Tam (10^{-9} to 10^{-6} M) which antagonize E_2 activation of ER, coexpression of HET/SAF-B (10 ng) significantly enhanced the antagonism by Tam.

We next addressed how increased overexpression of HET/SAF-B could affect the transcriptional activity of E_2-occupied ER. In the absence of ER, the addition of E_2 did not result in a significant change in basal activity of the construct, and HET/SAF-B had no effect on this basal activity. As expected, transfection of ER led to an approximately 6-fold increase of transcriptional activity in the presence of E_2. The coexpression of increasing amounts of HET/SAF-B (0–150 ng) led to a significant dose-dependent decrease in ER activity (Fig. 3B). A similar HET/SAF-B-mediated repression was also seen in Saos-2 cells transfected with ER (data not shown). Increasing concentrations of HET/SAF-B (100 and 250 ng) were also able to further enhance the antagonist activity of Tam, as shown in Fig. 3C.

Using the same transfection system in HepG2 cells, but incubating the cells in the presence of Tam alone, Tam acts as an agonist and can activate the ER. We therefore tested whether HET/SAF-B overexpression could affect Tam agonist activity. Figure 3D shows that Tam (10^{-8} M) caused a 2- to 2.5-fold increase in ER activity. Cotransfection with HET (100 ng) reduced this increase by 58%, while 250 ng HET completely abolished Tam agonist activity.

We next performed a series of additional control experiments (Fig. 4) to exclude a nonspecific repressor effect of HET/SAF-B. As shown in Fig. 3, A and B, basal activity of the ERE-tk-promoter was not inhibited by overexpression of HET/SAF-B. As expected, the deletion of the ERE sequence led to a tk-promoter construct that was also not affected by HET/SAF-B overexpression (Fig. 4A, *left bars*). In the same experiment the ERE-tk-promoter was inhibited by overexpression of HET/SAF-B (Fig. 4A, *right bars*), as shown previously (Fig. 3). As is common for transient transfection assays, the luciferase values were corrected for the values of a second cotransfected gene, which is, in our case, an SV40-promoter-driven β-galactosidase (β-gal) construct. Overexpression of HET/SAF-B did not change β-gal expression (Fig. 4B), thus representing another internal negative control. In several other experiments using other transcription factors and other reporter constructs, we again did not see a nonspecific repression by HET/SAF-B (detailed later).

The ER-DBD Is Necessary for the Repressive Effects of HET/SAF-B

Most nuclear receptors including ER share a typical domain structure: a Zn finger DBD is flanked by an N-terminal region that displays a constitutive activator function domain 1 (AF-1) and the C terminus containing the ligand-binding domain, heterodimerization domain, and ligand-dependent activation function domain 2 (AF-2). To delineate the importance of the DBD of ER on HET/SAF-B-mediated repression, we made use of chimeric constructs in which the ER-DBD (aa 178–257) was replaced by a GAL4-DBD and tested reporter activity on four copies of a gal4-responsive element upstream of luciferase (gal4-luc). As a negative control we included the GAL4DBD alone (GAL4DBD). As a positive control we transfected wild-type ER, HET/SAF-B, and the ERE-tk-luc. All constructs were cotransfected with HET/SAF-B into HepG2 cells. The data are presented in Fig. 5A (*left panel*) as fold over control of each construct, since the activity of the different GAL4DBD constructs varied over magnitudes. The relative luciferase units for the controls (no estrogen and no HET/SAF-B) were ER = 1437.0, gal4DBD = 1.2, and ER-gal4DBD = 26.1.

As expected, E_2 increased transcriptional activity from the ERE-tk-luc reporter construct, and the induction was repressed by coexpression of HET/SAF-B (Fig. 5A). Coexpression of the gal4DBD with the gal4-luc reporter construct resulted in basal activity that was not affected by E_2 treatment and was also not affected by coexpression of HET/SAF-B. Expression of both the AF-1 and the AF-2 domains fused to

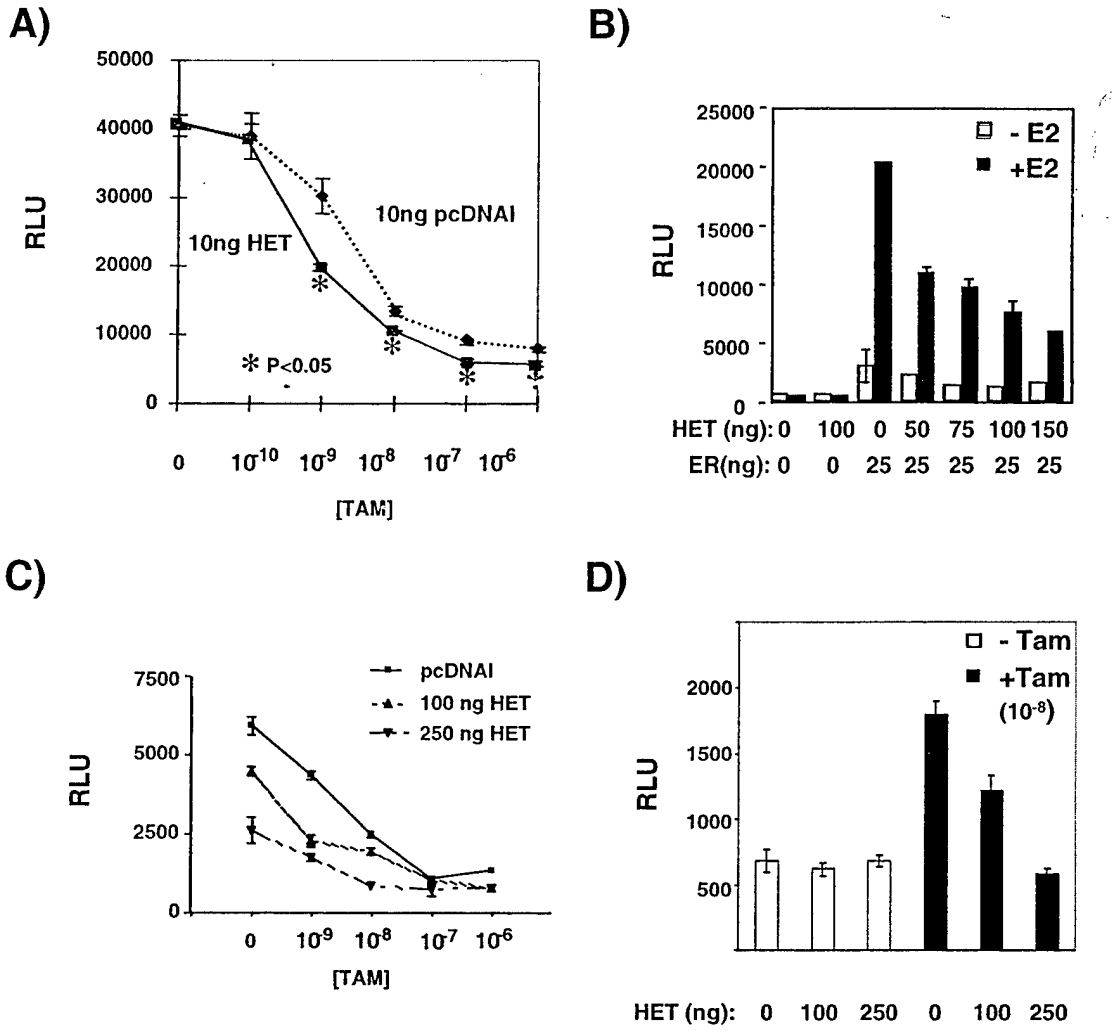

Fig. 3. Overexpression of HET/SAF-B Inhibits ER Activity

Cotransfection of the ERE-tk-luc (1 μg) reporter gene with expression vectors coding for HET/SAF-B and ER as indicated in HepG2 cells. Values are the mean ± SEM of triplicate wells, and the graphs are representative of at least three experiments each. A, Cells were transiently transfected with ER (25 ng) and 10 ng of pcDNAI or HET/SAF-B plasmids and incubated in the presence of 10^{-9} M E_2 and increasing amounts of Tam, as indicated (*, $P < 0.05$, *t* test). B, Cells were transfected with ER and HET/SAF-B plasmids as indicated. *Open bars* represent incubation in the absence of E_2, and *black bars* represent incubation in the presence of 10^{-9} M E_2. C, Cells were transiently transfected with ER (25 ng) and HET/SAF-B (100 and 250 ng) plasmids and incubated in the presence of 10^{-9} M E_2 and increasing amounts of Tam, as indicated. D, Cells were incubated in the absence of ligand (*open bar*) and in the presence of 10^{-8} M Tam (*black bars*), and transfected with ER (25 ng) and the indicated amounts of HET/SAF-B.

GAL4DBD (ER-GAL4DBD) behaved like wild-type ER with strong E_2 inducibility. However, HET/SAF-B did not repress activity while it did repress wild-type ER activity. In addition, HET/SAF-B was not able to repress activity of either AF-1 fused to GAL4DBD, or AF-2 fused to GAL4DBD (data not shown). Interestingly, while HET/SAF-B was not able to repress activity of the ER-GAL4DBD chimera, HET/SAF-B was still able to bind to this chimera as shown by coimmunoprecipitation (Fig. 5A, *right panel*). The binding of HET/SAF-B to ER GAL4DBD substantiates the earlier *in vitro* GST binding experiments indicating that HET/SAF-B can bind ER not only in the DBD, but also in the Hinge/AF2 region. Thus, while HET/SAF-B can bind ER-GAL4DBD, it cannot repress its activity, suggesting that the ERE-DBD is required for transcriptional repression.

To directly assess the importance of the ER-DBD in HET/SAF-B-mediated repression, we examined the effect of HET/SAF-B on the ER-DBD fused with a heterologous transcription factor (VP16). As a control we examined the effect of HET/SAF-B on VP-16 with a GAL4-DBD. As shown in Fig. 5B, the addition of increasing amounts of HET/SAF-B (50, 100, and 250 ng) did not affect the activity of VP16-GAL4 DBD on a GAL4 reporter construct. In contrast, HET/SAF-B caused a dose-dependent decrease of VP16-ER-DBD activity on a ERE-Luc reporter construct. Thus we can

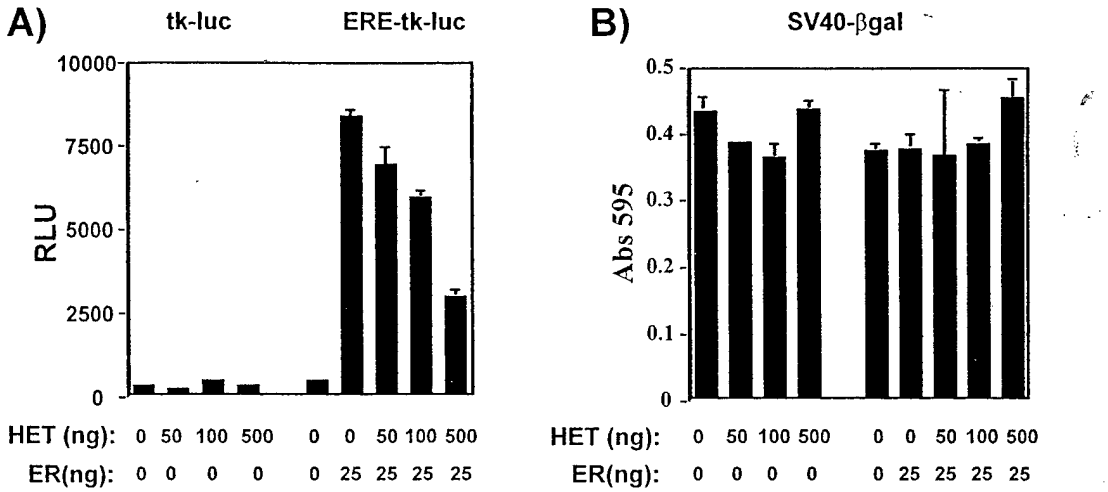

Fig. 4. HET/SAF-B-Mediated Repression of ER's Transcriptional Activity Is Not the Result of a General Repression Mechanism

Cotransfection of ERE-tk-luc and tk-luc reporter genes with expression vectors coding for HET/SAF-B and ER as indicated. *Bars* are the mean ± SEM of triplicate wells and each *graph* is representative of at least three experiments. A, HepG2 cells were transfected with expression plasmids for ER and HET/SAF-B, as indicated, and with the reporter constructs tk-luc and ERE-tk-luc, respectively. Cells were incubated in the presence of 10^{-9} M E_2. B, β-Gal values measured from the experiment shown in panel A.

conclude that the ER-DBD can mediate the HET/SAF-B transcriptional repression effect.

A simple explanation for the repressive effect of HET/SAF-B would be if HET/SAF-B bound to the ERE-DBD and blocked ER binding to DNA. To examine this possibility, we asked whether HET/SAF-B had an effect on the DNA binding properties of ER. First we confirmed that HET/SAF-B could not bind directly to an ERE sequence using gel-shift assays and *in vitro* transcribed and translated HET/SAF-B (data not shown). To then examine whether HET/SAF-B could inhibit ER binding to DNA, we used a promoter interference assay originally described by Reese and Katzenellenbogen (41) in which an ERE is inserted between the cytomegalovirus (CMV) promoter (containing the TATA box) and the start site of transcription of the chloramphenicol acetyl transferase reporter gene (CMV-ERE-CAT) (Fig. 5C). Constitutive expression of this reporter construct was inhibited by coexpression of ER (Fig. 5C). This inhibition occurs in the absence of ligand, but is enhanced by addition of E_2 or Tam, as previously shown by Reese and Katzenellenbogen. Coexpression of HET/SAF-B (10 ng) did not affect the activity of the reporter construct in the absence of ER and did not alter the ability of ER to inhibit reporter activity either in the absence or in presence of ligand. Overexpression of HET (250 ng) again did not affect the constitutive expression of the reporter construct in the absence of ER. However, this high concentration of HET/SAF-B actually increased the ability of ER to reduce reporter activity. This would suggest that binding of HET/SAF-B does not block the ability of ER to bind DNA, but rather that in the presence of high concentrations of HET/SAF-B more interference occurs.

DISCUSSION

Nuclear receptors are divided into three groups: steroid receptors, retinoic acid/thyroid receptors, and orphan receptors. A fundamental difference between steroid receptors and retinoic acid/thyroid receptors is that the latter are DNA-bound active repressors in the absence of ligand. An active role for corepressors such as N-CoR (8, 12) and SMRT (14) for the silencing activity of unliganded retinoic acid/thyroid receptors has been well established. Recent findings of disturbed corepressor interaction with mutated thyroid receptors in patients with resistance to thyroid hormone support the importance of corepressors for the normal action of agonist- and antagonist-bound receptors (42–44).

In contrast to retinoid/thyroid receptors, steroid receptors show little DNA binding activity in the absence of ligand and thus are thought to have no silencing ability. However, recently it has become clear that steroid receptors are also found in repressor complexes, particularly when the receptor is bound to antagonists, and that the antagonist function may in part be mediated by corepressors (15, 45, 46).

In the presence of the antiestrogen Tam, ER can still dissociate from heat shock proteins and bind to DNA, but its AF-2 domain activity is inhibited (47). It has been shown that ER can bind the corepressors N-CoR and SMRT (45, 46). While binding of these corepressors is constitutive under *in vitro* conditions (48), coimmunoprecipitation experiments have indicated that N-CoR binds to ER only in the presence of Tam (46). The specific role of N-CoR and SMRT in the antagonist effect of Tam is unclear, but more detailed studies

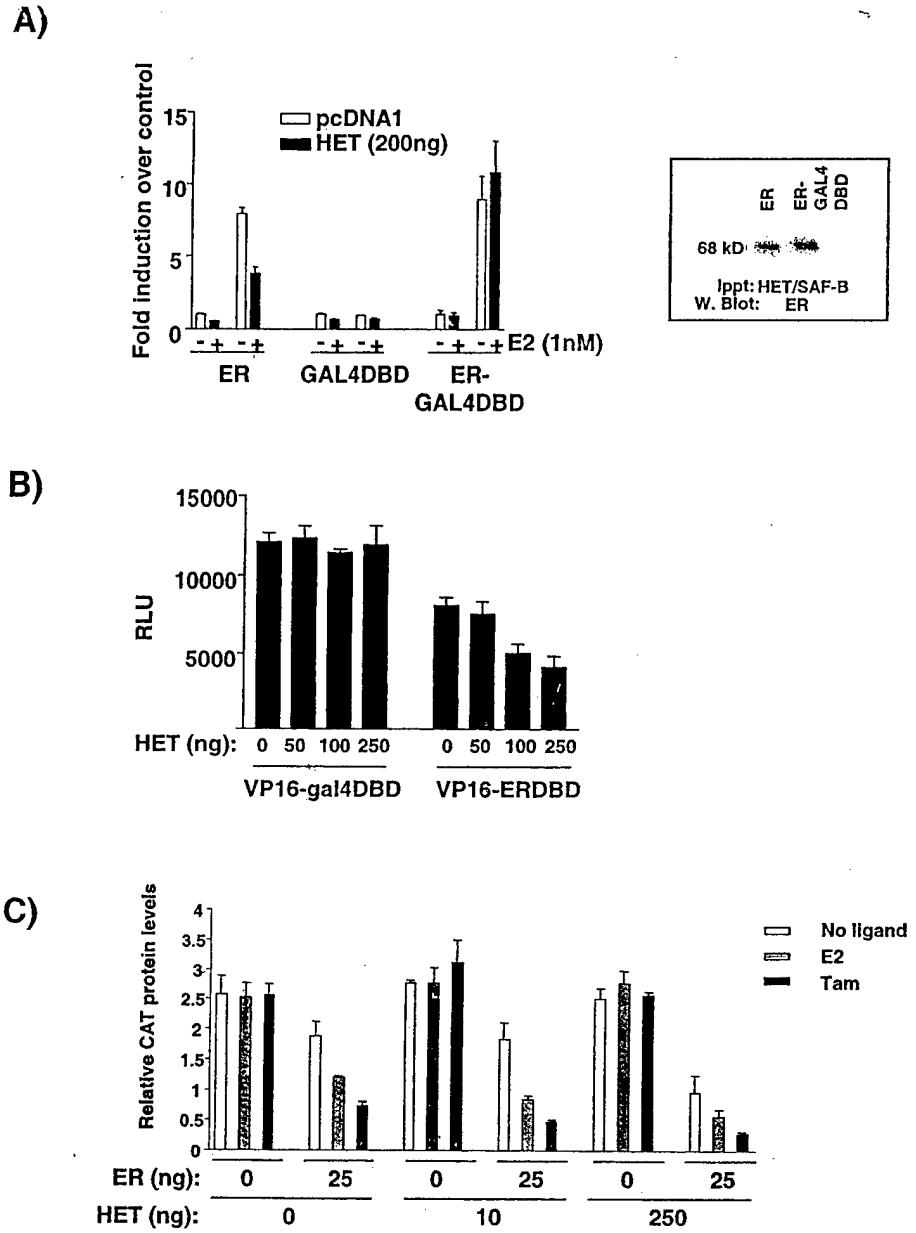

Fig. 5. ER-DBD Is Involved in HET/SAF-B's Mediated Repression of ER's Activity

HepG2 cells were transiently transfected with expression vectors as indicated. *Bars* are the mean ± SEM of triplicate wells and the *graphs* are representative of at least three experiments each. A, Cells were transiently transfected with various ER constructs (25 ng) and HET/SAF-B (200 ng), as indicated, and ERE-tk-luc and the Gal4-responsive promoter construct Gal4-luc, respectively. Since the activity of the different Gal4DBD constructs varied over magnitudes, the data are presented as fold over control calculated in relation to the activity of each construct seen in the absence of ligand. The *right panel* shows the result from an immunoprecipitation using lysates from COS-7 cells which were transiently transfected with expression plasmids for HET/SAF-B and ER-Gal4DBD. The cell lysates were immunoprecipitated with an anti-HET/SAF-B antibody, subjected to electrophoresis, and immunoblotted with an anti-ER antibody. B, Cells were transiently transfected with HET/SAF-B (as indicated), with 25 ng VP16-gal4DBD and VP16-ERDBD, and with the Gal4-responsive promoter construct Gal4-luc and ERE-tk-luc, respectively. C, Cells were transfected with 1 μg CMV-ERE-CAT, and ER, and HET/SAF-B as indicated. Cells were incubated in the absence of ligand (*white bars*), in the presence of 10^{-9} M E_2 (*gray bars*), and 10^{-7} M Tam (*black bars*).

have been performed concerning the agonist role of Tam. It has been shown that overexpression of N-CoR or SMRT can inhibit Tam's agonist activity (45, 46, 48). Additionally, reduction of N-CoR by microinjection of N-CoR-specific antibodies can convert Tam into a full ER agonist displaying activity similar to estrogen (46). However, no data at present confirm that N-CoR or SMRT are actually responsible or necessary for the

antagonist activity of Tam. More recently a novel ER-specific corepressor, termed REA, has been discovered (15). REA can potentiate the antiestrogenic effect of Tam, but when overexpressed at high levels also inhibits estrogen activation of the ER.

In the present paper we describe another protein association with ER, that of the nuclear matrix protein HET/SAF-B, which also has properties consistent with its being an ER corepressor. Under *in vitro* conditions, HET/SAF-B interacts with ER in the absence of ligand, although the association is increased by Tam. Furthermore, coimmunoprecipitation experiments show that the interaction between HET/SAF-B and ER is stronger in the presence of Tam. The ability of Tam to recruit HET/SAF-B, as well as N-CoR and REA, to ER suggests an active corepression mechanism, although this remains to be specifically proven. Due to the ability of N-CoR and REA to alter the agonist/antagonist activity of Tam, it has been proposed that the ratio of corepressor to coactivator levels can alter the response of the ER to estrogen or Tam (46). Our studies with HET/SAF-B certainly fit this model. We show that HET/SAF-B potentiates Tam's antagonist activity, while overexpression of HET/SAF-B at high levels inhibits E_2 and Tam agonist activities. Although HET/SAF-B's interaction with ER is weaker in the presence of E_2 as compared with Tam, we were able to detect repression of E_2-activated ER, just as described for N-CoR (45) and REA (15). This repression probably represents inappropriate binding between ER and HET/SAF-B in the presence of E_2 resulting from transient overexpression of HET/SAF-B. Under normal conditions we believe that Tam recruits HET/SAF-B to ER and that this association may be responsible, in part, for the antagonist effect of Tam.

Over the last couple of years it has become clear that transcriptional repression is an important strategy for fine regulation of growth, development, and differentiation. Despite the identification of corepressors, repressor motifs, and their targets, little is known about specific mechanisms of repression. Models that have been proposed include 1) interference with the formation or activity of the basal transcriptional machinery, 2) quenching of a transcriptional activator, and 3) induction of an inactive chromatin structure (reviewed in Ref. 49). It is likely that repression mediated by a corepressor like N-CoR is the result of a combination of these mechanisms. N-CoR is a large protein (270 kDa) that interacts with mSin3 and recruits histone deacetylase (21). Deacetylation results in conformational changes of the nucleosome structure, thereby limiting the accessibility of chromatin to the transcriptional machinery. In addition to its interaction with chromatin remodeling factors, Muscat *et al.* (50) have recently shown that N-CoR directly interacts with the basal transcription factors TFIIB, $TAF_{II}32$, and $TAF_{II}70$.

HET/SAF-B is a nuclear matrix protein with several recently described characteristics (31, 32, 35), which could be involved in repressive mechanisms. Like N-CoR, which interacts with multiple factors, HET/SAF-B is probably part of a multiprotein complex regulating ER activity. While the work described here does not directly address the mechanism of HET/SAF-B-mediated repression, some potential mechanisms can be considered.

First, HET/SAF-B has recently been shown to bind to the C-terminal domain of RNA pol II (35) in yeast two-hybrid systems. As hypothesized for the interaction of N-CoR and basal transcription factors (50), it is conceivable that HET/SAF-B locks the transcriptional initiation complex into a nonfunctional state. Second, HET/SAF-B-mediated repression might also involve changes in histone acetylation, since in our own preliminary experiments treatment with the histone deacetylase inhibitor trichostatin A (51) relieves HET/SAF-B-mediated repression (S. Oesterreich, unpublished results). Third, it is possible that the RNA-binding domain of HET/SAF-B is involved in repression. In addition to HET/SAF-B, other RNA-binding proteins, such as L7/SPA (45), hnRNP U (30), and more recently RNA itself, SRA (52), have been described as coregulators of nuclear hormone receptor action.

While HET/SAF-B shares some of the characteristics of other coregulators, it is possible that its repressive action results from its ability to associate with the nuclear matrix. The presence of specific binding sites for ER, also called "acceptor proteins", in the nuclear matrix was postulated several years ago after *in vitro* reconstitution experiments showed binding of the ER to the nuclear matrix to be saturable and of high affinity (53, 54). We have shown previously that 1) HET/SAF-B is associated with the nuclear matrix in biochemical fractionations (31); 2) HET/SAF-B and ER can both be cross-linked to scaffold attachment regions (55); and 3) ER can associate with the nuclear matrix as shown by direct visualization with a green fluorescent protein-tagged ER (56). In this paper we have described HET/SAF-B as a nuclear matrix protein/scaffold attachment factor that associates with the ER.

Finally, it is important to define the domains of the steroid receptors that are involved in the interaction with the nuclear matrix. Eggert *et al.* (30) demonstrated that the C terminus of the glucocorticoid receptor was sufficient for hnRNP U-mediated repression. In contrast, Tang *et al.* (57, 58) and van Steensel *et al.* (59) have shown that the DBD of the glucocorticoid receptor is required for interaction with the nuclear matrix, and that replacement of the GR-DBD by a Gal4DBD resulted in loss of hnRNPU-mediated repression. In a similar way, HET/SAF-B-mediated repression is lost when the ER-DBD is substituted by a Gal4DBD. Indeed, the ER-DBD is sufficient for HET/SAF-B-mediated repression. However, the repression is not a result of inhibiting ER's ability to bind to DNA.

In summary, our study has revealed that the nuclear matrix protein/scaffold attachment factor HET/SAF-B directly binds to ER and inhibits its activity. The binding is stronger in the presence of Tam, as compared with no ligand, suggesting that HET/SAF-B-mediated

corepression may be involved in the antiestrogenic effects of Tam.

MATERIAL AND METHODS

Plasmid Constructs and Chemicals

The cloning of the HET/SAF-B expression construct (31) and of the mammalian expression vector for full-length ER has been previously described (60). To generate an HA-tagged full length ER construct, ER was PCR-amplified (61) using the following primers: sense 5′-GCGAATTCATGGCTTACCCCTACGACGTC-CCCGACTACGCCATGACCATGACCCTCCAC-3′ comprising the HA-tag, and nucleotides 1–18 coding for the ER, and the antisense primer was 5′-GATGAATTCCTCAGACTGTGGC-AGGGAA-3′ comprising nucleotides 1770–1789 of the ER. The PCR product was cloned into pcDNA3.1/V5/His-TOPO (Invitrogen, Carlsbad, CA). To generate a GST-fusion protein, the full-length HET/SAF-B clone (31) was cloned into *Eco*RI sites of the pGEX-2TK gene fusion vector (Pharmacia Biotech, Piscataway, NJ). Bacterial expression vectors for GST-ER fusion proteins containing the AF1, DBD/Hinge, DBD, Hinge, and AF2/Hinge domains were generated by performing ligation reactions with the appropriate PCR products and *Eco*RI/*Bam*HI-digested pGEX-2TK. The positions of the PCR primers (linked to *Eco*RI or *Bam*HI sites) within the ERα cDNA (61) are: AF1 –sense (1–20) and antisense (519–540); DBD/Hinge –sense (519–540) and antisense (849–869); DBD –sense (513–533) and antisense (730–746); Hinge –sense (750–771) and antisense (844–863); AF2/Hinge –sense (756–775) and antisense (1769–1788). The AF1/DBD construct was a kind gift of Dr. S. Kato (62, 63). The gal4-luc construct (pfrluc) was purchased from Stratagene (La Jolla, CA). Constructs containing the activation domains (AF-1 and AF-2) of the ER fused to the gal4 DBD (amino acids 1–94) were a kind gift of Dr. O'Malley, and have been previously described (60). Briefly, the AF-1 domain of the ER was cloned upstream of the gal4DBD in pABgal94 (64) to create AF-1 gal4. The AF-2 domain of ER was cloned downstream of the gal4DBD to create AF2-gal4. Finally the AF-1 and AF-2 domains were cloned upstream and downstream, respectively, of the gal4DBD to create ER-gal4DBD. A construct containing a chimeric activator with the ER DBD and the activation region of VP16 was provided by Dr. P. Chambon and has been previously described (65). Finally, the ER-dependent promoter interference reporter plasmid was provided by Dr. B. Katzenellenbogen and has been described by Reese and Katzenellenbogen (41). The antiestrogen 4-hydroxytamoxifen (Tam) was a gift from Zeneca Pharmaceuticals (Macclesfield, UK). All other chemicals were purchased from Sigma (St. Louis, MO) unless stated otherwise.

Cell Culture and Transient Transfection

Human breast cancer cells (MCF-7), human hepatocyte carcinoma cells (HepG2) cells, and human osteosarcoma cells (Saos-2) were maintained in improved MEM (IMEM) supplemented with 5% FBS (Life Technologies, Gaithersburg, MD), 200 U/ml penicillin, 200 μg/ml streptomycin, 6 ng/ml insulin. COS-7 cells were maintained in DMEM +10% FBS, 200 U/ml penicillin, 200 μg/ml streptomycin, 6 ng/ml insulin. For reporter assays, cells were transiently transfected using Fugene (Roche Clinical Laboratories, Indianapolis, IN) following the manufacturer's protocol. One day before transfection cells were plated at 8×10^5 in six-well plates. For E_2 induction experiments the cells were plated in serum-free medium which consisted of phenol red-free IMEM + 10 mM HEPES, pH 7.4 + 1 μg/ml fibronectin (Life Technologies) + trace elements (Biofluids, Rockville, MD) + 1 μg/ml transferrin (Life Technologies). Cotransfections were performed using 1 μg reporter plasmid, 100 ng β-galactosidase (β-gal) expression vector, and HET/SAF-B and ER plasmids as indicated in the figure legends for each experiment. Twenty-four hours after transfection, the medium was replaced with serum free medium containing the appropriate ligand. Forty-eight hours later cells were washed twice with PBS, and luciferase activity was measured using the Luciferase kit from Promega Corp. (Madison, WI). β-gal activity was measured as described (31), and the luciferase activities were normalized by dividing by the β-gal activity to give relative luciferase units. For determining CAT activity, we used a CAT enzyme-linked immunosorbent assay from Roche Clinical Laboratories and followed the manufacturer's instructions. Values were corrected for protein concentrations and are presented as relative CAT activity. For transient transfections, triplicate samples were measured in each experiment, and the data are presented as the average ± SEM and are representative of at least three independent experiments. For coimmunoprecipitation experiments, COS-7 cells were plated at 0.6×10^6 into 10- cm dishes, and transiently transfected with 5 μg expression plasmids for HET/SAF-B and ER-HA. Twelve hours later the medium was replaced with phenol red-free IMEM + 5% charcoal-stripped serum and ligands as indicated in the figure legends. The cells were lysed 24 h later.

In Vitro Protein-Protein Interaction (GST Pull-Down)

Overnight cultures of *Escherichia coli* BL21 expressing the appropriate fusion constructs were diluted 1:10 in LB medium and incubated for 1 h. GST only or GST-fusion proteins were induced for 2.5 h with 0.1 mM isopropyl-β-D-thiogalactoside, followed by centrifugation, and resuspended at 1:100 in cell suspension buffer (1× PBS, 100 mM EDTA, pH 8.0, 0.1 mM phenylmethylsulfonyl fluoride, 0.2 μg/ml pepstatin, 0.2 μg/ml leupeptin, 0.2 μg/ml aprotinin, 0.2 μg/ml antipain). Cells were sonicated and then centrifuged for 10 min at 4 C, and 400 μg of crude *E. coli* bacterial extract proteins were incubated with 60 μl glutathione Sepharose 4B beads (50% slurry, Pharmacia Biotech) (1 h, 4 C). For the binding assay, the beads were incubated in IPAB buffer (150 mM KCl, 0.1% Triton X-100, 0.1% NP40, 5 mM $MgCl_2$, 20 mM HEPES, 20 μg/ml BSA, protease inhibitors), and ligand was added as indicated in the experiments. *In vitro* transcription-translation mixture (TNT kit, Promega Corp.) containing ^{35}S-methionine was programmed with HET/SAF-B and ER expression plasmids. Lysates (10 μl) were incubated with 60 μl equivalent amounts of GST proteins (as assessed by Coomassie staining) at 4 C for 1 h. The beads were washed three times with IPAB buffer without BSA. Bound proteins were eluted in SDS sample buffer, resolved by SDS-PAGE, and visualized by fluorography.

Generation of Anti-HET/SAF-B Monoclonal Antibodies

The peptide used for generation of a monoclonal antibody (mAb) to HET-SAF/B was identical to the peptide used to generate a polyclonal antibody described previously (31). The mAb was generated at the UTHSCSA Institutional Hybridoma Facility following methods described by Kohler (66) and Oi and Herzenberg (67). Briefly, spleen cells from two BALB/c female mice immunized subcutaneously three times with 50 μg keyhole limpet hemocyanin-coupled peptide in Freund's adjuvant were fused with NS-1 myeloma cell line. A 50% PEG solution was added in a drop-wise manner. The subsequent dilution was performed in selection media (hypoxanthine, aminopterin, thymidine-containing medium), and 10 days later supernatants were screened for relevant antibody using the A156 HET/SAF-B peptide coupled to an alternative carrier (BSA). Culture supernatant from clone 6F7 was purified using the ImmunoPure (A/G) IgG purification kit (Pierce Chemical Co., Rockford, IL).

Coimmunoprecipitation

MCF-7 cells were plated at 2×10^6 cells in 10-cm dishes. The next day the media was changed to media containing 5% charcoal-stripped FCS and ligand as indicated in the figure legends. Twenty-four hours later the cells were lysed in low-stringency (LS) buffer (PBS, 0.1% NP40, protease inhibitors), and HS buffer (20 mM Tris, pH 7.4, 50 mM NaCl, 1 mM EDTA, 0.5% NP40, 0.5% SDS, 0.5% deoxycholate, and protease inhibitors), followed by sonication. Sodium tetrathionate (50 μM) was added to the lysis buffer since it is known to selectively stabilize interactions between hormone receptors and the nuclear matrix (68); however, its addition is not essential for coimmunoprecipitation of ER and HET/SAF-B. The lysate was precleared with 50 μl protein G-agarose for 30 min at 4 C, and then incubated overnight with 7 μl HET/SAF-B mAb at 4 C. Protein G agarose was added for another 4 h, and the beads were pelleted and washed three times with the indicated buffer. For immunoprecipitation of HA-ER we precleared the lysates with 20 μl protein A-agarose, incubated with 5 μl HA antibodies (Babco, Richmond, CA) overnight, and finally added 20 μl protein A-agarose. Bound proteins were eluted in SDS sample buffer, subjected to SDS-PAGE, and analyzed by immunoblotting (see below). For quantification, the scanned image was analyzed using NIH Image 2.0. The background intensity was subtracted from the intensity of the ER band in the immunoprecipitation, and this arbitrary number was divided by the intensity for the ER band in the immunoblot. The result is represented as an arbitrary number of the intensity ratio of immunoprecipitated ER to immunoblotted ER in the lysates.

Immunoblotting

Proteins were resolved on 8% SDS-PAGE and electrophoretically transferred to nitrocellulose. The membrane was blocked in PBS/0.1% Tween 20 (PBST) + 5% milk for 1 h at room temperature. HET/SAF-B, ER (6F11, Novacastra, Newcastle upon Tyne, UK), and HA-(Babco, Richmond, CA) antibodies were diluted at 1:1000, 1:100, and 1:1000, respectively, in PBST + 5% milk. After incubation for 1 h, the membrane was washed six times for 5 min each time with PBST, the membrane was incubated with horseradish peroxidase-linked anti-mouse IgG at 1:1000 (Amersham Pharmacia Biotech, Arlington Heights, IL) in PBST + 5% milk, washed six times for 5 min each time, and the signal was developed using enhanced chemiluminescence according to the manufacturers instructions (Pierce Chemical Co.).

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HET/SAF-B Overexpression Causes Growth Arrest and Multinuclearity and Is Associated with Aneuploidy in Human Breast Cancer[1]

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ABSTRACT

HET/SAF-B was originally cloned as a nuclear matrix protein that bound to matrix attachment regions and as a transcriptional repressor of the small heat shock protein hsp27. In addition, we have found recently that HET/SAF-B is also a corepressor of estrogen receptor activity. Estrogen receptor has a very well-described role in breast cancer, and aberrant expression of nuclear matrix and heat shock proteins has also been implicated in breast tumorigenesis. Therefore, we asked whether HET/SAF-B itself could be important in breast cancer. Toward this goal we examined its expression in breast cancer cell lines and asked whether HET/SAF-B can affect breast cancer cell proliferation. Finally, we studied HET/SAF-B expression in clinical breast cancer samples.

HET/SAF-B protein and mRNA were detected at varying levels in all of the eight breast cancer cell lines examined. Using a number of different approaches to modulate the level of HET/SAF-B protein in the cell, we found that HET/SAF-B levels are inversely correlated with cell proliferation. In addition, transfection of HET/SAF-B fused to the green fluorescent protein led to the formation of multinucleated cells not observed in cells transfected with green fluorescent protein alone, suggesting that this effect is a direct result of HET/SAF-B overexpression. Western blot analysis of HET/SAF-B in 61 human breast tumors revealed widely varying levels of HET/SAF-B expression, with some tumors (16%) lacking any detectable HET/SAF-B. Statistical analysis showed that high HET/SAF-B expression in these tumors was associated with low S-phase fraction and with aneuploidy, consistent with our results from transfection experiments in tissue culture cells. We conclude that HET/SAF-B plays an important role in breast cancer, and we discuss possible mechanisms of the involvement of HET/SAF-B in cell proliferation and division.

INTRODUCTION

HET/SAF-B was originally cloned as a protein binding to matrix/scaffold attachment regions (1) and as a NMP[3] binding to the hsp27 promoter in human breast cancer cells (2). Subsequently, it was shown to bind to the COOH-terminal domain of RNA polymerase II and to a subset of serine/arginine-rich RNA processing factors (SR proteins) and to function in mRNA splicing (3). This suggests that HET/SAF-B is involved in the formation of a "transcriptosomal" complex, bringing transcription and mRNA processing together. These macromolecular complexes have been shown previously to be associated with the nuclear matrix (4, 5).

The nuclear matrix consists of a protein-RNA network that is involved in structural organization of DNA within the nucleus, thereby controlling important regulatory processes such as transcription and DNA replication (reviewed in Ref. 6). Not surprisingly, many NMPs have been shown to be important in cell transformation. The NMP pattern of expression shows significant differences between normal and cancer tissue in bladder (7), colon (8), head and neck (9), prostate (10), and breast (11). Consistent with this, various NMPs were found to have potential as prognostic markers for cancer add (12, 13). Additionally, a role for the nuclear matrix in steroid hormone action was postulated many years ago (14–18) but only recently have specific NMPs been characterized that directly bind to hormone receptors and modulate their activity (19). For example, recently, the gluticorticoid receptor-interacting protein GRIP 120 has been identified as the NMP hnRNPU (20). We have shown recently that the NMP HET/SAF-B regulates the activity of the estrogen receptor (21).

HET/SAF-B binds to the ER and functions as an ER corepressor. In this way, HET/SAF-B is similar to several other

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[3] The abbreviations used are: NMP, nuclear matrix protein; hsp, heat shock protein; ER, estrogen receptor; GFP, green fluorescent protein; FBS, fetal bovine serum; SFM, serum-free medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CMV, cytomegalovirus; RPA, RNase protection assay; FACS, fluorescence-activated cell sorter.

recently identified ER-interacting proteins, REA (repressor of estrogen receptor activity; Ref. 22), SMRT (the silencing mediator of retinoid and thyroid receptors; Refs. 23 and 24), and NcoR (nuclear receptor corepressor; Ref. 25), all of which also act as corepressors. Because estrogen is one of the most potent mitogens for breast cancer cells and is a known risk factor for breast cancer, a role of HET/SAF-B in estrogen action implies a role in ER-positive breast cancer cell growth control. Alternatively, it is also possible that HET/SAF-B can act as a transcriptional repressor independent of ER by interacting with other transcription factors. It has been shown that known steroid receptor-interacting proteins such as the coactivator SRC1 (26, 27), which was originally cloned as a steroid receptor cofactor, also mediates transactivation by other transcription factors including AP1 (28), serum response factor (28), nuclear factor-κB (29), cyclic AMP-responsive element binding protein, and signal transducers and activators of transcription (30). More recently SRC1 has also been found to bind to p53 and potentiate its transactivation, whereas two other ER coactivators, amplified in breast cancer (AIB1) and *Xenopus* steroid receptor coactivator (xSRC-3), were found to repress p53-mediated transactivation (Lee *et al.*, 1999). This suggests that these factors might have important and distinct roles in tumorigenesis independent of their function as a steroid hormone receptor regulator.

HET/SAF-B is involved in a number of cellular processes that are associated with tumorigenesis. These include its role in the repression of hsp27, which has been shown to positively regulate breast cancer cell proliferation (31), as well as it role as a NMP and as an ER corepressor. Therefore, we have set out to analyze whether HET/SAF-B plays a role in breast cancer. Here we report that overexpression of HET/SAF-B causes growth inhibition and multinuclearity in cultured cells. Consistent with these findings from tissue culture, HET/SAF-B expression is associated with lower proliferation but also with aneuploidy in human breast tumor specimens. Thus, as predicted, HET/SAF-B plays a role in breast tumor behavior. Possible mechanism(s) will be discussed in more detail.

MATERIALS AND METHODS

Plasmid Constructs and Chemicals. The cloning of the HET/SAF-B expression construct in pcDNA1 has been described previously (2). To generate an antisense construct, the full-length *Eco*RI-digested HET/SAF-B construct was cloned into pcDNA1 in the antisense direction, which was verified by sequencing. For the RNase protection assay, an *Apa*I-*Eco*RV HET/SAF-B fragment (99–443 bp) was cloned into pGEM5Zf(+) (Promega Corp., Madison, WI) and restriction-digested with *Xho*I (200 bp), and the probe was made using a T7 polymerase. The 36B4 probe has been described previously (32). A GFP-HET/SAF-B fusion protein with GFP positioned at the COOH-terminal of HET/SAF-B was cloned by ligating the full-length HET/SAF-B into the *Eco*RI site of pEGFP-C3 (Clontech, Palo Alto, CA). To generate an inducible HET/SAF-B construct, HET/SAF-B cDNA1 was subcloned from pcDNA1 using *Eco*RI and cloned into the unique *Eco*RI site in pUHD10–3 (33) to generate pUHDHET. The orientation was confirmed by sequencing. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless stated otherwise.

Cell Lines and Tumors. Breast cancer cell lines MCF-7/MG, MDA-MB-468, MCF-7, MDA-MB-231, MCF-7/BK, MDA-MB-330, ZR-75, and T47D, along with CHO-K1 (Chinese hamster ovary) cells and T24 (human bladder carcinoma), were maintained in IMEM supplemented with 10% FBS, 2 mM glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin. NIH3T3 (mouse embryo) and 293 (transformed human embryonic kidney) cell lines were kept in DMEM (Life Technologies, Inc., Grand Island, NY), with the same supplements as IMEM. SFM consisted of IMEM + 10 mM HEPES (pH 7.4), 1 μg/ml transferrin, 1 μg/ml fibronectin, 2 mM glutamine, 50 IU/ml penicillin, 50 μg/ml streptomycin, and trace elements (Biofluids, Rockville, MD). The clinical breast tumor specimens for the Western blot study were obtained from the National Tissue Resource maintained by our Breast Cancer Specialized Program of Research Excellence. These specimens were originally sent by hospitals throughout the United States to Nichols Institute Research Laboratories in San Juan Capistrano, CA, for routine measurements of steroid receptors and cell cycle analyses by flow cytometry. The flow cytometric assays were performed using methods described previously (34).

Transfections, Cell Growth, and Cell Cycle Analysis. All transfections were performed using Lipofectamine (Life Technologies, Inc.) or Fugene (Roche Molecular Biochemicals, Indianapolis, IN). Transient transfections were analyzed 48 h after transfection. To establish stable cell lines, NIH3T3 cells were cotransfected with pcDNA1 only or HET/SAF-B-pcDNA1 (2) and pSVneo, and transfected clones were selected in 1000 μg/ml G418.

For growth analysis, cells were plated in quadruplicate at 2500 cells/well in a 96-well plate. The next day (day 0), cell number was assessed by MTT assay as described previously by us (35). Cells were then incubated in SFM or medium with 10% FBS, and cell number was determined at days 2, 4, and 6.

For colony formation assays, MCF-7/MG cells were transfected with 20 μg of pCDNA1 vector control or HET/SAF-B-pcDNA1 sense and antisense, respectively, along with 1 μg of pSVneo. After 3 weeks incubation in 400 μg/ml G418, colonies were stained with 1% crystal violet.

293 cells, which display very high transfection efficiency, were used for proliferation assays measuring [^{3}H]thymidine incorporation into DNA. Cells (8×10^4) were plated in triplicate in six-well plates and transfected on day 2 with increasing amounts of pcDNA1 or HET/SAF-B- pcDNA1 antisense constructs. On day 4, the cells were incubated for 1 h with 1 μl/ml [^{3}H]thymidine (Amersham; 1 mCi/ml). After washing in cold PBS and cold 5% trichloroacetic acid, the cells were kept on ice for 30 min in the presence of 5% trichloroacetic acid and finally lysed in 0.5 M NaOH.

For generation of inducible HET/SAF-B-expressing cells, we used the tetracycline inducible expression system, which has been described in detail previously (33, 36). The tetracycline inducible MDA-MB-453rtTA cells were given to us by Dr. Douglas Yee (University of Minnesota). Briefly, cells were stably transfected with a plasmid (pUHD172–1-neo) expressing a protein termed rtTA (VP16 linked to a tetracycline binding protein). Stable clones were selected in 1000 μg/ml G418, expanded, and then tested for expression of rtTA by transient transfection with a reporter plasmid (pUHD16–3) consisting of

seven tetracycline operator sequences upstream of a luciferase gene. Treatment of cells with doxycycline (an analogue of tetracycline) at 1 μg/ml for 24 h indicated inducible luciferase expression (2–10-fold) in a number of clones. We used the clone with the highest inducibility (MDA-435rtTA1) for transfection with an expression plasmid containing HET/SAF-B under the control of a tetracycline-inducible CMV promoter (pUHDHET). After transfection, these cells were selected in 600 μg/ml hygromycin and analyzed for inducible HET/SAF-B expression by Western blot analysis.

For the analysis of cell cycle distribution, cells were harvested, washed with PBS, fixed in 70% ethanol, and stored at −20°C. Immediately before analysis on a FACS STAR PLUS (Becton Dickinson, San Jose, CA), propidium iodide and RNase were added to the cell pellet to final concentrations of 0.1 and 0.5 mg/ml, respectively. Data were analyzed using CellQuest software.

The expression of HET/SAF-B throughout the cell cycle was investigated in T24 bladder carcinoma cells grown in IMEM containing 5% FBS by first growing the cells to confluence and then leaving them for 3 days to arrest in G_0-G_1 (37). The cells were then subcultured into 10-cm plates and plated at a density of 1×10^6 per well. Cells lysates were produced at different time points after subculture by first washing the cells in PBS and then lysing them in high salt buffer [50 mM Tris-HCl (pH 7.8), 0.2 mM EDTA, 0.4 M NaCl, 10% glycerol, and 1% $NaPO_4$] containing Protease Inhibitor Cocktail Tablets (Roche Molecular Biochemicals, Indianapolis, IN) used at the concentration suggested by the manufacturer. T24 cells were blocked in G_2-M using nocodazole. Cells were grown in 15-cm culture dishes and blocked by the addition of nocodazole at 40 ng/ml for 18 h. Cell lysates were produced as described above. HET/SAF-B protein levels in the cell lysates were determined by Western blotting using 50 μg of total protein and our monoclonal HET/SAF-B antibody (21).

RNA and Protein Analysis. The RPAs were performed as described previously (31) using a HET/SAF-B-specific probe as well as a probe for 36B4 as a loading control. For Western blot analysis, cell pellets were resuspended in 5% SDS or high salt buffer and sonicated, and 50 μg of total protein were analyzed by Western blotting using HET/SAF-B antibody as well as a polyclonal antibody against the p85 subunit of PI3K (Upstate Biotechnology, Lake Placid, NY) as a loading control. For the quantitative Western blot analysis of human tumors, each gel contained 50 μg of MCF-7 SDS extract as an internal standard. The HET/SAF-B bands were quantitated by densitometric scanning using NIH Image 1.6 software, and the levels were calculated in arbitrary units by the ratio of the integrated densitometry signal in the tumor sample relative to the internal standard on each gel. For the detection of GFP-HET/SAF-B fusion protein, an anti-GFP antibody was used at a 1:1000 dilution (Clontech, Palo Alto, CA).

Statistical Analysis. All statistical analyses were performed using SAS (Version 6.11; SAS Institute, Cary, NC) running on a Sun Microsystems SparcServer 1000. Relationships between HET/SAF-B expression and S-phase fraction and between HET/SAF-B and ER expression were analyzed using Spearman's rank correlation coefficients. The relationship between HET/SAF-B expression and ploidy was analyzed using a *t* test.

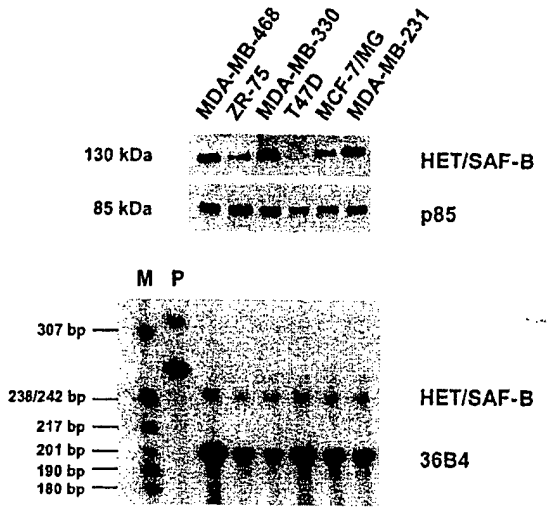


Fig. 1 HET/SAF-B expression in breast cancer cell lines. For the Western blot (*A*), 50 μg of SDS-protein extracts were loaded onto 6% SDS-PAGE, transferred to nitrocellulose, and blotted with HET/SAF-B- and p85-specific antibodies. For the RPAs (*B*), 20 μg of RNA from the cell lines used in the Western blot were used, and the protected HET/SAF-B fragment of 240 bp is shown. 36B4 was used as a loading control.

RESULTS

Expression of HET/SAF-B in Breast Cancer Cell Lines. Renz and Fackelmayer (1) have shown previously that *HET/SAF-B* is a ubiquitously expressed gene. Northern blot analysis using a variety of different human cancer cell lines and different tissues detected HET/SAF-B mRNA in all analyzed samples. To see how it is expressed in various breast cancer cell lines, we performed Western blot analysis using the HET/SAF-B antibody and a p85 antibody as a loading control (Fig. 1). HET/SAF-B protein levels varied between cell lines, with the highest expression in MDA-MB-231, ranging to almost undetectable levels in ZR-75 cells. The subsequent RPA (Fig. 1*B*) indicated that ZR-75 cells do express HET/SAF-B mRNA. Thus, although *HET/SAF-B* is a ubiquitously expressed gene, the levels in breast cancer cell lines vary.

Overexpression of HET/SAF-B in Tissue Culture Cell Lines and Effect on Cell Growth. To establish the relationship between HET/SAF-B expression and cell proliferation, we attempted to transfect HET/SAF-B into cell lines. A number of initial efforts to isolate stable breast cancer cell lines that constitutively overexpressed HET/SAF-B were unsuccessful; after transfection with HET/SAF-B-pcDNA1, some drug-resistant clones formed, but none survived further passaging in culture. In contrast, we were able to select several hundred control clones transfected with pcDNA1 alone. These results indicate that HET/SAF-B either inhibits proliferation or is toxic to the cells. To circumvent this problem, we used a tetracycline-inducible

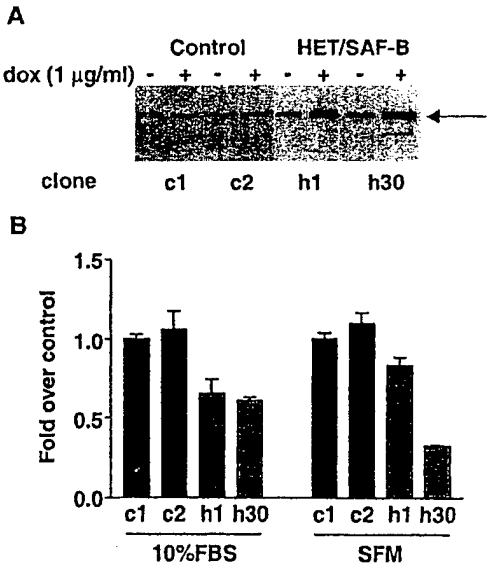


Fig. 2 Inducible expression of HET/SAF-B and growth inhibition in MDA-MB-435rtTA cells. *A*, MDA-MB-435rtTA cells containing a HET/SAF-B plasmid under control of a tet-inducible CMV promoter were induced with doxycycline for 24 h, lysed in 5% SDS, and analyzed by Western blotting using HET/SAF-B-specific antibodies. *B*, cells were plated in 96-well plates in quadruplicate in 10% serum (■) and in SFM (▨), and doxycycline was added the next day. On day 5, MTT assay was performed, and the data are presented as percentage growth inhibition as compared with control cells; *bars*, SD.

system in MDA-MB-435 cells (MDA-435 rtTA1). These cells were transfected previously with an inducible transactivator (etoposide linked to a tetracycline binding protein) and show 5–10-fold induction of reporter gene activity in the presence of the inducer doxycycline. We therefore cloned the HET/SAF-B cDNA downstream of a tetracycline-regulated CMV promoter and generated inducible HET/SAF-B clones. We were able to isolate two inducible clones, which showed increased HET/SAF-B expression when cells were stimulated with doxycycline for 24 h (Fig. 2*A*). We performed MTT growth assays to measure proliferation rate in these clones as compared with control clones and detected a significant decrease in cell number when cells were stimulated with doxycycline (Fig. 2*B*). The HET/SAF-B overexpressing cells showed growth inhibition in 10% serum as well as in serum-free medium. However, further passaging of those cells resulted in loss of inducibility of HET/SAF-B expression. Because we detected a slight leakiness of the system in transient assays, *i.e.*, expression of HET/SAF-B in the absence of inducer (data not shown), we suggest that the clones were lost because of a low overexpression of HET/SAF-B, even in the absence of doxycycline.

Because NIH3T3 cells are known to be less sensitive to overexpression of exogenous genes as compared with breast cancer cells, we attempted to generate stable HET/SAF-B transfectant overexpression clones with these cells. We could not detect any HET/SAF-B by Western blot in parental NIH3T3 cells, which could be attributable either to very low expression or to the inability of the antibody raised against human HET/SAF-B to recognize murine HET/SAF-B. Just as in breast cancer cells, drug-resistant colonies formed after transfection in NIH3T3 cells, but most did not survive passaging, although we were able to select a high number of control clones transfected with the empty vector pcDNA1. Finally, we were able to generate one HET/SAF-B overexpressing clone (#25), as confirmed by Western blotting (Fig. 3*A*). Anchorage-dependent growth assays (MTT assay) showed that the HET/SAF-B-overexpressing clone grew much slower than two vector-alone control clones, either in SFM or in 10% FBS (Fig. 3*B*). Furthermore, cell cycle analysis confirmed that the slower growth of the HET/SAF-B-overexpressing clone was accompanied by a decrease in S-phase (15.6–4.0%). Thus, HET/SAF-B overexpression results in growth inhibition. This growth inhibition seems to be independent of ER, because it can be observed in ER-negative cells.

Overexpression of a GFP-HET/SAF-B Fusion Protein in Tissue Culture Cell Lines. As a final approach to generate HET/SAF-B-overexpressing clones, we used a GFP-HET/SAF-B fusion protein for our transfection studies. We hypothesized that using the fluorescently tagged HET/SAF-B would improve our screening procedure, because only fluorescent clones would be picked, expanded, and analyzed for overexpression. GFP-HET/SAF-B is functional, because it was able to corepress ER activity (data not shown), similar to our findings using the HET/SAF-B construct (21).

We transfected MDA-MB-435 cells using the GFP-HET/SAF-B construct and GFP only as a control. Of 20 fluorescent clones that were transfected with GFP alone, all 20 were still brightly fluorescent after keeping them in culture for 4–6 weeks (data not shown). However, of 120 fluorescent GFP-HET/SAF-B clones that we originally isolated, only 4 were still fluorescent after expanding them (2–3 weeks). In two of those clones, HET/SAF-B localized to the cytoplasm (data not shown), which has not been described before and which might represent an "escape mechanism" from the growth-inhibitory and/or toxic effects of HET/SAF-B overexpression. The other two clones showed only very faint fluorescence, in only approximately 1–3% of the cells, and HET/SAF-B was not detectable by Western blotting using the HET/SAF-B antibody, presumably because of the low level of expression in a small number of cells. However, a very weak signal could be detected using an anti-GFP antibody on Western blots (data not shown). We did not perform any growth assays with theses clones because the expression of the exogenous HET/SAF-B was considerably lower than endogenously expressed HET/SAF-B, so that significant effects were unlikely to be observed. Thus, although the use of a GFP-HET/SAF-B fusion protein allowed us to perform a more efficient primary screen of the colonies, subsequently we were again unable to keep HET/SAF-B-overexpressing cells in culture.

We did, however, notice an obvious morphological change in the GFP-HET/SAF-B-expressing MDA-MB-435 cells as compared with the GFP-expressing cells (Fig. 4*A*). Many GFP-HET/SAF-B cells were polynucleated, with some cells having as many as 20 nuclei. We did not detect any polynucleated cells in the control GFP-transfected cells. We confirmed this result in transiently transfected CHO-K1 cells (data not shown), where

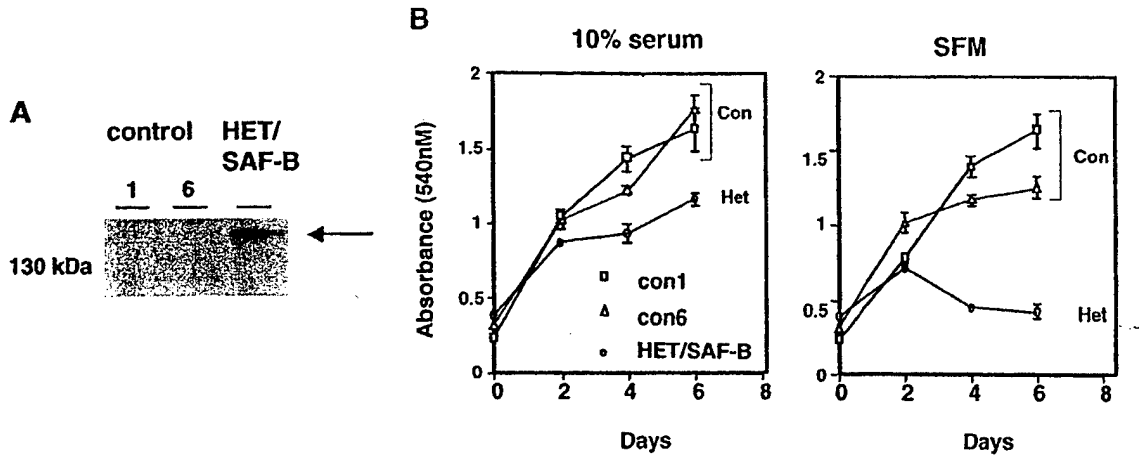
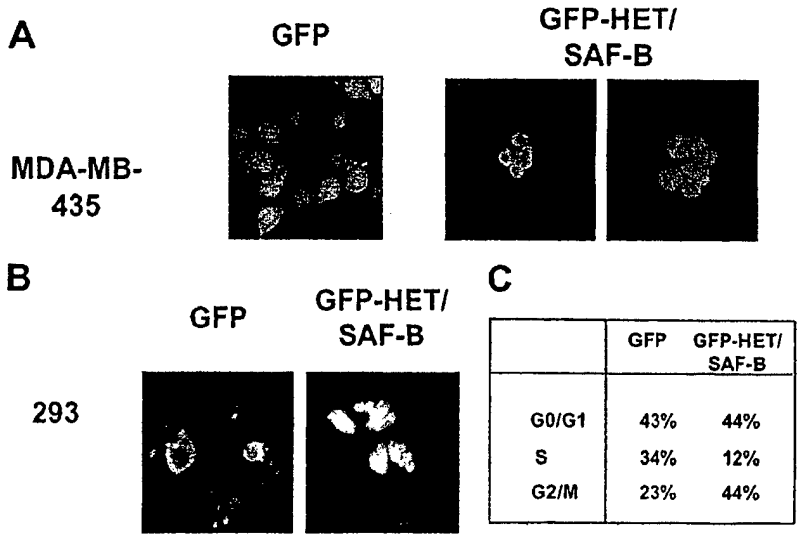


Fig. 3 Overexpression of HET/SAF-B and growth inhibition in NIH3T3 cells. *A*, for the Western blot, 50 μg of SDS-protein extracts were loaded onto 6% SDS-PAGE, transferred to nitrocellulose, and blotted with HET/SAF-B-specific antibodies. *B*, cells were plated in quadruplicate in 96-well plates in 10% serum or SFM, and MTT assays were performed on the next day (day 0) and on day 2, 4, and 6. *Bars*, SD.



	GFP	GFP-HET/ SAF-B
G0/G1	43%	44%
S	34%	12%
G2/M	23%	44%

Fig. 4 Generation of multinucleated cells after GFP-HET/SAF-B transfection. *A*, MDA-MB-435 cells were transfected with GFP only or with GFP-HET/SAF-B. Cells were observed using a FITC filter on an Olympus CK40 fluorescence microscope. Representative pictures of one control clone (GFP) and two GFP-HET/SAF-B clones are shown. *B*, 293 cells were transiently transfected with GFP or GFP-HET/SAF-B, and representative pictures were taken. *C*, transfected 293 cells were sorted on a FACS, and DNA histogram analysis was performed on fluorescent cells only.

again we detected many polynucleated cells in the GFP-HET/SAF-B-overexpressing cells but not in the GFP cells. Finally, we repeated the transient transfection in 293 cells, which display very high transfection efficiency. As seen in MDA-MB-435 and CHO-K1 cells, we again observed many polynucleated cells among the HET/SAF-B-overexpressing cells. Depending on the cell line used, we detected multinucleated cells in 1–5% of the cells. We analyzed the cell cycle distribution of the transfected 293 cells by sorting the fluorescent cells and subjecting them to DNA histogram analysis (Fig. 4*D*). As shown previously (in the NIH3T3 transfection in Fig. 3), the number of cells in S-phase was decreased, from 34% in control cells to 12% in GFP-HET/SAF-B-overexpressing cells. We also observed a block in G_2-M in the HET/SAF-B-overexpressing cells (23–44%). Thus, overexpression of GFP-HET/SAF-B was associated with multinuclearity and significant changes in cell cycle.

Because overexpression of HET/SAF-B seemed to block cells in G_2-M, we asked whether HET/SAF-B protein levels vary through the cell cycle. To answer this question, we used T24 human bladder carcinoma cells that can be easily synchronized by contact inhibition as described previously (37). They reenter the cell cycle upon replating at a lower dilution. Breast cancer cells do not synchronize upon confluence but can be synchronized by withdrawal of serum. However, reentry into the

A

Stage	Cell Cycle Distribution (in %)						
G0/G1	52.8	92.3	87.9	58.9	3.4	28.4	0.7
S	36.2	3.6	6.6	40.5	54.8	34.6	35.4
G2/M	10.9	4.0	5.4	0.5	41.7	37.1	63.7

B

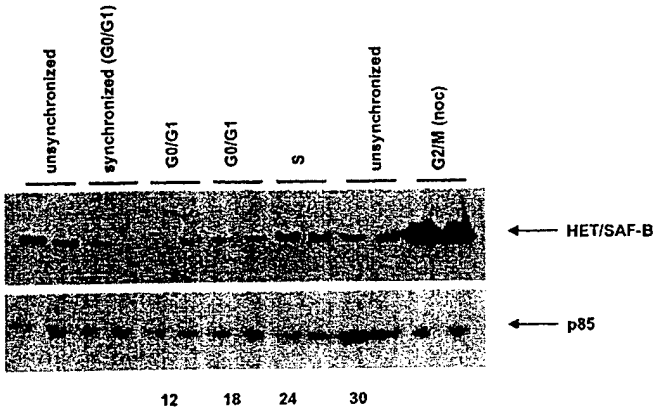


Fig. 5 Cell cycle-dependent expression of HET/SAF-B. *A*, the cell cycle distribution for the cell populations used in the Western blot in *B*. *Columns* correspond to the *lanes* on the Western blot below. *B*, Western blot of HET/SAF-B in T24 cells either synchronized or blocked in G_2-M by nocodazole. The lanes represent extract from duplicate plates. T24 cells were synchronized, and samples were taken at 8, 12, 24, and 30 h after synchronization to investigate HET/SAF-B expression. T24 cells were also blocked in G_2-M using nocodazole at 40 ng/ml. HET/SAF-B protein levels were determined using our HET/SAF-B antibody. p85 was used as a loading control.

cell cycle by serum stimulation may produce artifactual results because serum stimulation may affect HET/SAF-B levels or phosphorylation. Thus, we used synchronized T24 cells. In addition, we analyzed T24 cells that were blocked in G_2-M by treatment with nocodazole. Cell cycle analysis was performed by FACS, and as expected, we were able to obtain cells synchronized in G_0-G_1, G_1-S, and G_2-M (Fig. 5*A*). Subsequent Western blot analysis demonstrated that HET/SAF-B was expressed throughout the cell cycle, but the levels of HET/SAF-B protein increased during S-phase and peaked in G_2-M (Fig. 5*B*). The expression of HET/SAF-B in nocodazole-treated cells confirmed the increase of HET/SAF-B expression in G_2-M. Thus, HET/SAF-B protein levels are at their highest in G_2-M, which is the stage at which overexpressed HET/SAF-B blocks cells.

Transient Modulation of HET/SAF-B Levels in Tissue Culture Cell Lines. As shown in the above experiments, it was difficult to stably integrate HET/SAF-B into the genome of various cell lines. Thus, as a final approach to prove the effect of HET/SAF-B on proliferation, we used a transient tissue culture assay in breast cancer cells commonly used by other investigators (38) to demonstrate negative effects of genes on growth. In this assay, cells are transfected with the gene of interest and a selection marker and then grown in selection media until colonies can be stained and counted. We transfected MCF-7/MG cells with pSV-neo plasmid and empty vector only (pcDNA1) or HET/SAF-B cDNA in sense or antisense orientation. Transfection of the antisense construct resulted in decreased HET/SAF-B expression at both RNA and protein levels (data not shown). The cells were selected for resistance to G418, and after 3 weeks, colonies were stained with crystal violet (Fig. 6*A*). The number of colonies was dramatically reduced after transfection of HET/SAF-B sense cDNA, whereas antisense transfection had no significant effect compared with pcDNA1

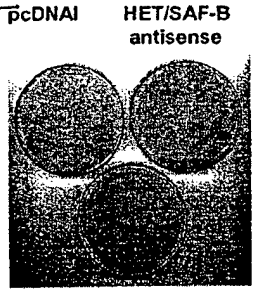


Fig. 6 Transient modulation of HET/SAF-B levels in MCF-7 cells. Colony formation assay in MCF-7 breast cancer cells is shown. MCF-7/MG cells were transfected with pcDNA1 vector control or with pcDNA1-HET/SAF-B in antisense or sense orientation, along with pSVneo, and incubated in G418 for 3 weeks. After staining with crystal violet, pictures were taken.

only. The inhibition of colony growth after HET/SAF-B overexpression confirms its growth inhibition. We were, however, surprised by the finding that antisense transfection did not increase colony formation in this experiment, and this could have several reasons. For instance, it is possible that we did not decrease the endogenous levels enough for generation of a phenotype in the transfected MCF-7 cells. Therefore, we repeated the transfection of the antisense DNA in 293 cells, which are known to display very high transfection efficiency. Determining [^{3}H]thymidine incorporation into DNA as a direct measurement of cell proliferation, we were able to detect a dose-dependent increase in the proliferative index after transfection

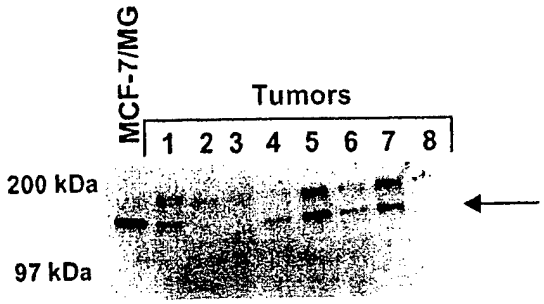


Fig. 7 Expression of HET/SAF-B in clinical breast cancer samples. A Western blot using 50 μg of SDS extracts from breast tumors and HET/SAF-B-specific antibodies is shown. In the *left lane*, an SDS-extract from MCF-7 breast cancer cells was loaded as a positive control. *Arrow*, position of HET/SAF-B.

of the antisense construct (data not shown), thus again supporting our other data describing HET/SAF-B as a negative growth regulator.

Expression of HET/SAF-B in Clinical Breast Cancer Specimens. Lastly, we asked whether in tumor samples we could detect an association of HET/SAF-B with proliferative markers in a way reflective of our findings from tissue culture experiments. Therefore, we analyzed HET/SAF-B expression in human breast cancer specimens and correlated this with proliferation rate as measured by S-phase fraction. We measured HET/SAF-B levels in 61 primary breast tumors by Western blot (a representative blot is shown in Fig. 7) and found that, as in the cell lines, HET/SAF-B protein content varied widely. Some tumors expressed high amounts (*e.g.*, no. 5), some moderate (*e.g.*, no. 6), and in 10 tumors (16%), no HET/SAF-B could be detected (*e.g.*, no. 8), even after prolonged exposure of the film. The same extracts were analyzed for histone H3 levels,[4] and samples that were HET/SAF-B negative still showed abundant histone H3 expression. Thus, we can exclude artifacts such as nonspecific protein degradation. We quantified HET/SAF-B protein levels by densitometry, and statistical analysis revealed a trend toward a negative correlation with S-phase fraction. The correlation was only borderline significant (Spearman's rank correlation, -0.22; $P = 0.08$), but the sample size was relatively small, and a larger analysis is planned to verify this correlation. We also detected an association between HET/SAF-B and ploidy; high HET/SAF-B levels were associated with increased aneuploidy ($P = 0.021$). HET/SAF-B levels did not correlate with ER levels (Spearman's rank correlation, -0.064; $P = 0.62$), although as shown in a number of previous studies, we were able to detect a correlation between high ER levels and low S-phase (Spearman's rank correlation, -0.23; $P = 0.025$). Thus, in human breast tumors, HET/SAF-B protein expression varied widely, and higher levels were associated with aneuploidy. Also, we detected a trend toward a negative association with proliferation, which is consistent with our findings from tissue culture experiments.

DISCUSSION

HET/SAF-B is a NMP that was cloned in our laboratory as a negative regulator of hsp27 expression (2) and in the laboratory of Renz and Fackelmayer (1) as a scaffold/matrix attachment site binding protein. The nuclear matrix was postulated many years ago to interact with nuclear hormone receptors (14–18), but only recently have specific NMPs been identified that bind directly to hormone receptors and modulate their activity (19). Because ER, like HET/SAF-B, is also involved in regulating hsp27 expression (39), we asked whether HET/SAF-B interacts with this receptor and modulates its activity and found that HET/SAF-B acts as an ER corepressor. Here we show more generally that HET/SAF-B is a growth inhibitor independent of its interaction with ER, blocking the cell in G_2-M, and in some situations causing multinuclearity.

We provide several lines of evidence that HET/SAF-B affects proliferation: (*a*) breast cancer cells with inducible HET/SAF-B expression grew significantly slower than their appropriate control clones; (*b*) NIH3T3 cells overexpressing HET/SAF-B also showed a significant decrease in growth; and (*c*) transient overexpression and underexpression of HET/SAF-B results in decreased colony formation and increased cell proliferation, respectively. Although interpretation of results from a single overexpressing NIH3T3 clone is limited because of the possible unpredictable effect of integration, the parallel evidence from HET/SAF-B-inducible MDA-MB-435rtTA cells and additional results from transient transfection assays in MCF-7 and 293 cells allow us to conclude that HET/SAF-B overexpression results in growth inhibition. In addition, in cultured cells as well as in clinical breast tumors, HET/SAF-B protein levels were inversely correlated with S-phase fraction, which is a direct measure of proliferation rate. In the present relatively small sample of breast tumors ($n = 61$), this correlation did not quite reach statistical significance ($P = 0.08$), and we are currently designing a larger study to address how well HET/SAF-B correlates with S-phase and other prognostic factors and whether HET/SAF-B levels could predict clinical outcomes of breast cancer patients. We have also shown that down-regulation of endogenous HET/SAF-B can lead to a higher growth rate, consistent with HET/SAF-B being a negative growth regulator, the absence of which may lead to excessive growth in tumors. Indeed, we have found that some breast tumors did not express HET/SAF-B protein at a detectable level.

The growth-inhibitory effects of overexpressed HET/SAF-B in ER positive cells can be readily explained by HET/SAF-B being an ER corepressor. It is feasible that HET/SAF-B suppresses estrogen-dependent transcriptional pathways related to breast epithelial cell proliferation, thus resulting in growth arrest. It is of interest to mention that the breast cancer susceptibility gene *BRCA1* was shown recently to inhibit ER activity in transient transfection assays (40).

The exact mechanism of estrogen-induced proliferation is yet to be defined, but we think that HET/SAF-B could play a role. When cells are primed to respond to estrogen in G_0-G_1 and in G_1-S-phase transition (41), HET/SAF-B levels are at their

[4] C. K. Osborne, unpublished results.

lowest. In contrast, when cells do not respond to estrogen (in M phase), HET/SAF-B levels are high. Overexpression of HET/SAF-B, for instance as a result of our transfection studies, results in high HET/SAF-B levels at all parts of the cell cycle, including the phases when cells are primed to respond to estrogen. This presumably leads to inappropriate ER corepression, thus resulting in growth arrest.

As described by us (21) and others (1, 3), HET/SAF-B clearly has other functions, independent of its ER corepressor activity. These characteristics or other functions of HET/SAF-B yet to be defined might explain the growth-inhibitory effect of HET/SAF-B in ER-negative cells. For instance, hsp27 has clearly been shown to be associated with increased breast cancer cell growth, so that its down-regulation by HET/SAF-B could result in growth inhibition. It is also likely, just as discovered for other steroid receptor cofactors (42), that HET/SAF-B does not interact exclusively with ER. Indeed, our preliminary data[5] indicate that HET/SAF-B can also inhibit the activity of other members of the steroid receptor as well as the retinoic acid/thyroid receptor families. Again, this regulation of other proteins besides the ER could explain the observed ER-independent effects.

HET/SAF-B has also been shown to bind to the COOH-terminal domain of RNA polymerase II and to a subset of serine/arginine-rich RNA processing factors (SR proteins; Ref. 3). This suggests that HET/SAF-B is involved in the formation of a transcriptosomal complex, bringing transcription and pre-mRNA processing together. The role of HET/SAF-B in this complex might be to prevent processing of mRNA transcripts, which would be consistent with the function of HET/SAF-B as a transcriptional repressor. Such an activity has been shown for CstF-50, which also binds to RNA polymerase II and is thought to prevent the processing of mRNA transcripts containing error (43). Overexpression of HET/SAF-B may disrupt the balance in the interaction with these splicing proteins, leading to a decrease in RNA processing and possibly resulting in growth arrest.

Finally, the attachment of HET/SAF-B to the nuclear matrix should be mentioned. The nuclear matrix organizes DNA into loop domains, the bases of which contain the S/MAR DNA sequences. It is the scaffold attachment factors such as HET/SAF-B that bind these sequences, thus connecting the chromatin to the NMP structures. The high levels of HET/SAF-B seen in G_2-M may also reflect its role in the packaging of chromatin for mitosis. When cells are in G_2-M, most transcription is repressed, and there is a dramatic increase in HET/SAF-B protein levels, which suggests that HET/SAF-B could be a major factor in the general repression of transcription at this phase of the cell cycle. The effect on cell proliferation when HET/SAF-B is overexpressed in both ER-positive and ER-negative cell lines may be the result of high intracellular HET/SAF-B levels throughout the cell cycle, leading to disruption of the organization of the transcriptionally active chromatin normally seen in G_0-G_1 and S-phase, thus producing transcriptionally inactive chromatin as seen in G_2-M. This repression then leaves cells without the appropriate protein machinery to continue through the cell cycle and they become blocked.

In addition to the growth-inhibitory effects of overexpressed HET/SAF-B, our experiments with GFP-HET/SAF-B show that overexpression causes cells to be multinucleated. Furthermore, in breast tumors, HET/SAF-B was strongly associated with aneuploidy. Multinuclearity and aneuploidy are two of the most common features of tumor cells, but the exact molecular basis for these phenotypes is unknown (44, 45). Because HET/SAF-B overexpression results in a block in G_2-M, one could imagine that overexpressed HET/SAF-B somehow disrupts mitosis, either directly by altering the condensation of chromatin or indirectly by repressing genes involved in spindle formation and cytokinesis.

In summary, we have provided evidence that the NMP HET/SAF-B plays several roles in human breast cancer. Our current studies are aimed at further identifying the mechanism(s) of HET/SAF-B-mediated growth inhibition and multinuclearity.

[5] S. Oesterreich, unpublished results.

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